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INVESTIGATION OF THE METHOD TO DETERMINE CARBOXYHAEMOGLOBIN IN BLOOD

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ABSTRACT

The current method for determining blood carboxyhaemoglobin (COHb) concentration by a GC technique has been critically evaluated and compared to spectrophotometric analysis methods. The major sources of bias in the method accuracy have been found to be in the measurement of blood volume and in the separate determination of total haemoglobin (Hb) concentration in the blood. The method is estimated to have a coefficient of variation (CV) of 6.2% over the range of 0.5 to 20 vol % COHb. The major source of method variability is from small changes in GC flow rate. The bias in the method was determined by comparison to UV spectrophotometric techniques.

Standardization procedures which have been examined are the use of a 500 ppm CO in nitrogen gas mixture and CO-saturated blood solutions as external standards. Also, methods of saturating blood with CO have been examined, with the use of an impinger or tonometer held at 37°C giving the most effective results. A modified analysis procedure based on the results obtained has been implemented and validated using CO-saturated blood. Tonometer saturated blood was found to contain 100.9 $\pm$ 4.6% COHb, whereas impinger saturated blood was found to contain 99.8 $\pm$ 4.8% COHb by this modified method.

The modified GC analytical procedure was compared to CO-oximetry and estimates from alveolar CO measurements and found to be accurate over the range of 0.5 to 20% COHb saturation.

Studies of long-term storage of CO-containing blood have been carried out and have revealed that the COHb concentration remains stable for up to nine days if the blood is stored anaerobically at refrigerator temperatures. Blood containing 10% or less COHb has been found to be stable up to 60 days.

INTRODUCTION

The determination of carboxyhaemoglobin (COHb) in blood using a gas chromatographic procedure has been carried out at DCIEM for many years (1). However, requirements for accurate determination of blood COHb over the range of 0.5 vol% to 20.0 vol% have pointed to the need for a critical examination of the current method for blood COHb determination using gas chromatography. Areas of concern include the method of standardization, variability at each stage of the analysis and whether alternative standardization procedures are available. These factors are examined in the current study.

BACKGROUND

A wide variety of techniques have been developed for the measurement of COHb in blood. These include gasometric methods (2, 3), calorimetric methods (4), gravimetric methods (5), gas chromatography (6-9), UV-visible spectrophotometry (10-12), electrochemistry (13, 14), infrared spectrometry (15, 16), photochemistry (17) and magnetic circular dichroism spectroscopy (18). The methods that have been developed may be roughly subdivided into two groups:

1. Methods in which the CO is liberated from COHb in the blood and measured either physically or chemically.

2. Methods in which the COHb fraction is estimated directly in the blood.

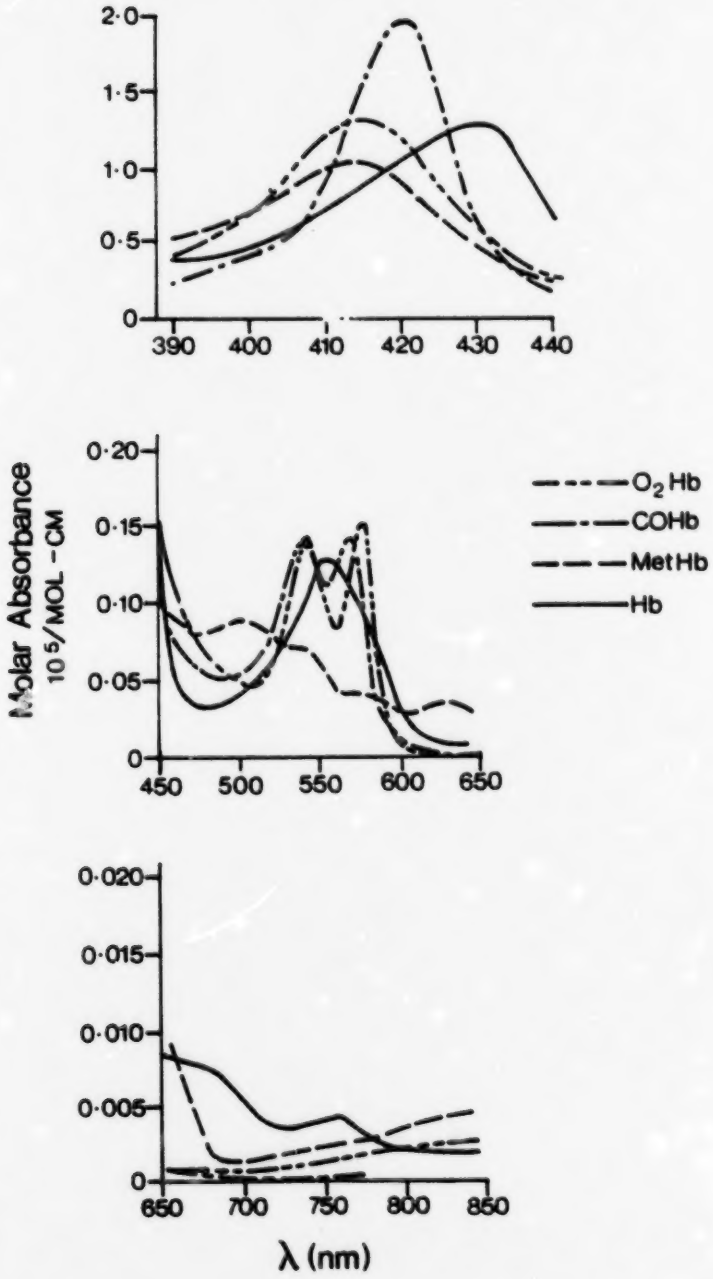
The most widely used techniques are either variations of the gas chromatographic method in which CO is detected by GC after liberation from COHb in the blood or variations of the spectrophotometric method in which COHb is determined in relation to other Hb species in the blood. COHb concentrations can also be estimated from measurements of the CO concentrations of alveolar air using a relationship established by Stewart (19).

The spectrophotometric determination of COHb is dependent on the difference between the COHb absorption curve and the absorption curves for other forms of haemoglobin (Hb) which may be present in the blood, particularly oxyhaemoglobin (HbO_2), reduced haemoglobin (HHb) and methaemoglobin (also referred to as haemiglobin) (MetHb). Three spectral regions have been used to measure differences in absorption maxima and isosbestic points for calculation of the concentrations of the above species in blood. These are illustrated in Figure 1 and are composed of:

1. A region of the ultraviolet (UV) which lies between 390 nm and 440 nm. The various Hb species show a single sharp peak in this region with millimeter extinction coefficents (E_{mM}) of about 120 to 150 $cm^{-1}.mM^{-1}$ per heme (5);

FIGURE 1

Spectra for the Major Haemoglobin Species



2. The visible region from 500 nm to 650 nm, where one or two characteristic maxima are found for each Hb derivative. These maxima are somewhat broader and about ten times less intense than those seen in the UV region; and

3. A region of the red to near infrared from about 650 nm to 1000 nm, with featureless spectra but fairly large relative differences in extinction coefficients, although these are approximately an order of magnitude lower than those in the visible region.

Most of the methods described in the literature calculate concentrations of Hb species present in a blood sample from extinction data measured at wavelengths from either the UV region or the 500 to 650 mm region of the visible spectrum. Numerous combinations of wavelengths can be used for measuring and calculating COHb concentrations in relationship to the other Hb species present. A number of these wavelength combinations have been summarized and discussed by Maehly (5).

A spectrophotometric technique which is widely used because of its speed and good accuracy over most of the range of COHb saturation is CO-oximetry. A CO-oximeter measures sample absorbance at four or six wavelengths (depending on the manufacturer) in the visible region (to avoid sample dilution) and calculates the concentration of each major Hb derivative present using preset extinction coefficients, as well as total Hb present (20, 21). Heinemann et al. (12) compared different spectrophotometric methods against CO-oximetry and found the five-wavelength method of Commins and Lawther (22), which uses wavelengths in both the UV (414, 420 and 426 nm) and visible (559 and 575 nm) regions, to be most precise. Zijlstra and co-workers have described several spectrophotometric methods for the simultaneous determination in blood of different Hb species, including COHb (20, 23, 24, 25).

The main advantages of spectrophotometry are speed of analysis, particularly using CO-oximetry, and its ability to measure several Hb species, including COHb, simultaneously. For analyses making use of the UV region, the necessary high dilution of the blood samples can cause considerable dissociation of CO from COHb, although Small et al. (26) outline a method for correcting for this. This loss of CO by dilution is almost exclusively due to replacement of CO by O_2 dissolved in the dilution liquid. If the HbO_2 plus COHb is reduced by sodium dithionite ($Na_2S_2O_4$) before dilution, the loss of COHb is small, as the reducing agent takes up the dissolved oxygen. However, addition of NaS_2O_4 can result in sulphaemoglobin (SHb) formation, which has been shown to cause interference with MetHb and COHb determination (20), although a correction can be applied (20, 23). Excessive amounts of MetHb in the blood can also interfere with COHb determination (27). This becomes important in analyses of aged blood, although a correction can again be applied (27).

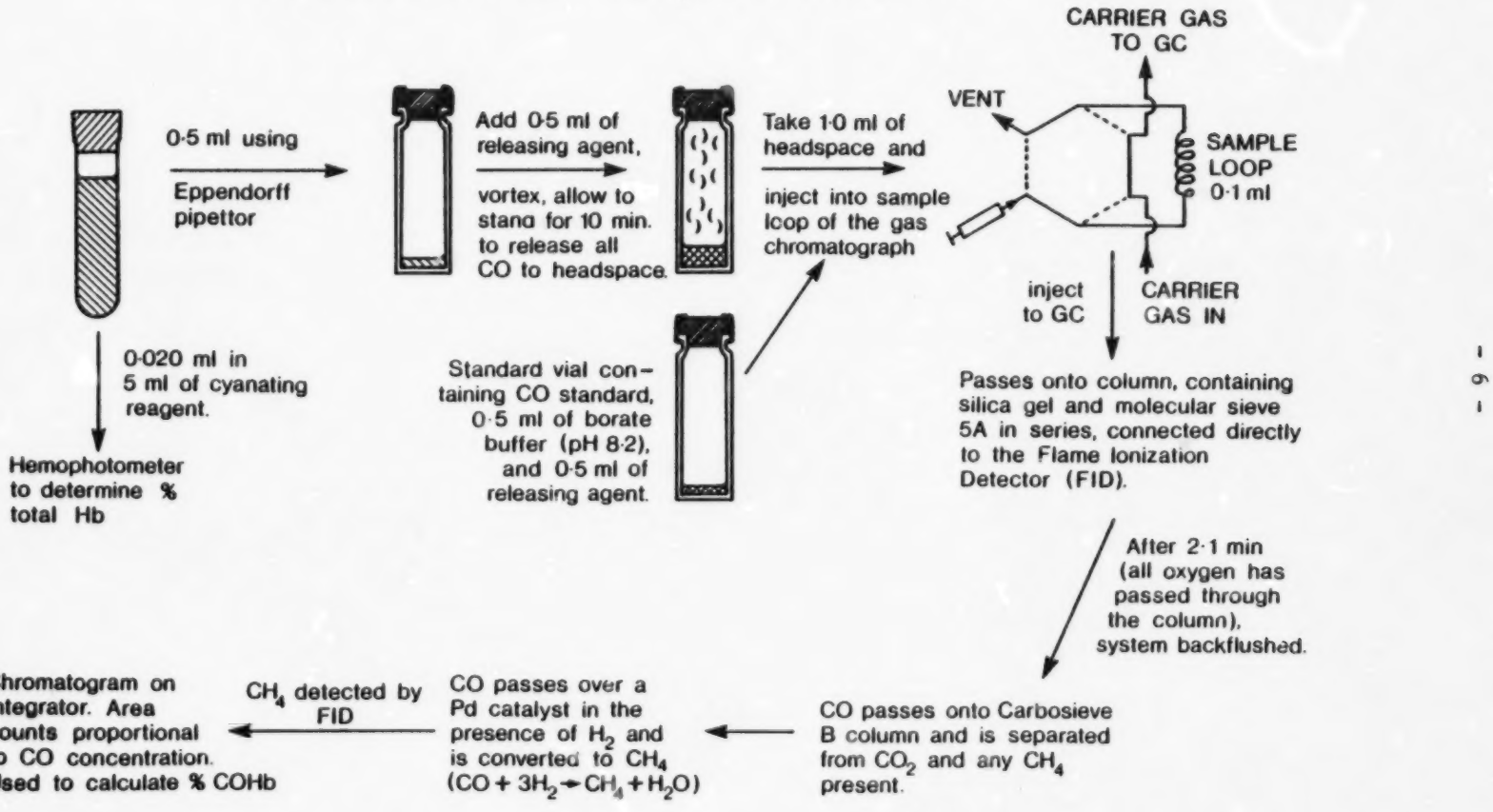
Other disadvantages of the spectrophotometric method include the fact that the maxima observed are not rigid constants but vary from sample to sample. This is due to variation in different types of haemoglobin between individuals (5). Accuracy of wavelength setting and reproducibility from determination to determination becomes especially important because of such variation, particularly at isosbestic points where the slope of absorbance versus wavelength may be changing rapidly. One study attempts to circumvent this problem by taking higher derivatives of the spectra to minimize interferences (28). One disadvantage of this method is that specialized instrumentation is required. There is evidence that CO-oximetry gives artificially high values of COHb at COHb concentrations below about 5%. This can be inferred from comparisons by Wigfield et al. (18) who show that the method of Commins and Lawther (22) gives high values of % COHb at concentrations below 10%, whereas Heinemann et al. (12) show excellent correlation between the Commins and Lawther method and CO-oximetry. Also, Guillot et al. (9), in their comparison of gas chromatography and CO-oximetry showed that the regression line more closely agreed with the line of identity if comparisons below 2% COHb were removed from the regression.

Gas chromatographic (GC) procedures have not been as widely used as spectrophotometry in determining COHb concentrations in blood, probably because of the complexity of apparatus required and the requirement that CO be liberated from the blood before analysis. However, the accuracy of GC over all COHb concentrations and freedom from interferences from other Hb derivatives make this method an excellent alternative to spectrophotometry. The first attempts to measure blood CO by GC were made in the late 1950s (29). Subsequent developments in the 1960s and the 1970s (30-33) refined the accuracy and precision of the method as well as the method of CO extraction. A major increase in method sensitivity was realized by using a nickel or palladium catalyst to convert CO to methane (CH_4) for subsequent detection by flame ionization (34). This method was adapted to the analysis of CO in blood by Baretta et al. (7). The method has also been improved recently by the use of a head-space gas chromatograph to measure released CO (9).

Although these developments have refined the accuracy, sensitivity and precision of the technique, the basic methodology has changed little. The method used at DCIEM is illustrated in Figure 2. An aliquot of blood is sealed into a reaction vial and a reagent added to haemolyze the blood cells and release CO from the COHb in the cells. The headspace containing CO is then either sampled and injected to the GC or the whole headspace is swept into the GC and subsequently analysed. The haemolyzing and releasing agent usually contains a surfactant such as Sterox SE or Triton X-100 to break down the blood cells and an oxidizing agent (normally potassium ferricyanide) to break down the haemoglobin, releasing CO. Variations include the amount of blood used, the method of haemolysation, the method of introduction to the GC and the type of column and detector used. Stevenson and

FIGURE 2

Schematic of GC Analysis Procedure.


0·5 ml using
Eppendorff pipettor
0·020 ml in 5 ml of cyanating reagent.
Hemophotometer to determine % total Hb
Add 0·5 ml of releasing agent,
vortex, allow to stand for 10 min. to release all CO to headspace.
Standard vial containing CO standard, 0·5 ml of borate buffer (pH 8·2), and 0·5 ml of releasing agent.
Take 1·0 ml of headspace and
inject into sample loop of the gas chromatograph
CARRIER GAS TO GC
VENT
SAMPLE LOOP 0·1 ml
inject to GC
CARRIER GAS IN
Passes onto column, containing silica gel and molecular sieve 5A in series, connected directly to the Flame Ionization Detector (FID).
After 2·1 min (all oxygen has passed through the column), system backflushed.
CO passes onto Carbosieve B column and is separated from CO_2 and any CH_4 present.
CO passes over a Pd catalyst in the presence of H_2 and is converted to CH_4 ($CO + 3H_2 \rightarrow CH_4 + H_2O$)
CH_4 detected by FID
Chromatogram on integrator. Area counts proportional to CO concentration. Used to calculate % COHb

co-workers, for example, have used as little as 2 ul of blood and detected released CO using a gas reduction detector (35, 36).

A necessary part of any GC method for determining blood COHb is the determination of total Hb in the blood sample. This is normally carried out separately by reacting an aliquot of blood with Drabkin's reagent (37) to form cyanmethaemoglobin (CNMetHb) and measuring absorbance at 540 nm spectrophotometrically (38). Instruments are available, called haemophotometers, which are calibrated to read out directly in %Hb using this method. The method usually specified a reaction time of 10 minutes before reading. However, at higher levels of COHb, a significant error can occur because the conversion of COHb to MetHb is slower at these higher levels (39). Thus, reaction times of up to 3 hours can be necessary at very high levels of COHb before complete conversion to CNMetHb has occurred. The reaction time can be accelerated by increasing the concentration of oxidising agent (potassium ferricyanide) in the Drabkin's reagent (39).

Standardization of methods used to determine COHb concentrations has been based on several different principles. For spectrophotometric methods, the spectrophotometer is first calibrated using filters which pass light of accurately-known wavelength. Then blood standards are prepared containing 100% HbO_2 (0% COHb) and 100% COHb. These standards are used to estimate molar absorptivities at the chosen analytical wavelengths. A calibration curve is then prepared using solutions prepared by mixing the above blood standards in accurately-known proportions. Thus, for the most accurate determinations, calibration is dependent upon the degree of COHb saturation achieved in the (nominally) 100% COHb blood standard. MetHb cannot react with CO, thus, any amount present in the blood will prevent 100% saturation being obtained. Other haemoglobin species in the blood may have a similar effect. However, the effects are likely to be small at COHb saturations below 20%. Difficulties arise when estimating blood COHb at very low levels, where the difference in molar extinction coefficients at a particular wavelength between the low COHb containing solution and the 0% COHb reference standard becomes very small and precision and accuracy of determination become degraded.

Gas chromatographic methods can also be calibrated against standard gas mixtures containing CO (7). This method should be more accurate than using CO-saturated blood, provided that complete release of CO from the sample prior to analysis is achieved. Kupferschmidt and Perrigo (40) and Blackmore (41) have examined the factors affecting CO release from blood, and found that acidic potassium ferricyanide solution combined with a haemolyzing agent such as Sterox SE or Triton X-100 is most efficient for this purpose.

An alternative method of standardizing CO release from blood has been described by Kijkhuizen *et al.* (24). The method uses potassium ferricyanide and Sterox SE to release CO from blood, and the CO is then

converted to CO_2 by passing over a cupric oxide (CuO) catalyst held at 400°C. The resultant CO_2 is subsequently titrated in a solution of barium chloride ($BaCl_2$) in a water/butanol mixture held at pH 10 using a sodium hydroxide (NaOH) solution of known strength. The method is calibrated using accurately metered amounts of pure CO. The method has been used to calibrate spectrophotometric procedures (23, 24), but could be applied to the calibration of GC methods.

METHODOLOGY

A series of experiments were carried out to define the accuracy and precision of the current analysis procedures, to determine the adequacy of the standardization procedures, and to examine alternative standardization procedures. The methods used are outlined below.

1. Determination of Accuracy and Precision

The current analysis protocol used to detemine COHb in blood is outlined in Annex A. Sources of variability and error in the method were examined as follows:

a. Vial Volume - Ten clean, dry reaction vials of 20 ml nominal capacity, complete with grey rubber septa and seals were weighed to the nearest 0.01 g using an analytical balance. The vials were filled with deionized water, sealed and reweighed. The weight of deionized water used gave an estimate of the vial volume. This was carried out on each batch of vials used.

b. Blood Volume - Twenty clean, dry 20 ml reaction vials complete with septa and seals were weighed to the nearest 0.1 mg using an analytical balance. 100 ul of whole blood were pipetted into each of 10 vials, 500 ul of whole blood were pipetted into each of the 10 remaining vials using Eppendorf single delivery pipettes, the vials sealed and reweighed. The weight difference was used to calculate the blood volume delivered by each pipette using a blood density of 1.0564 $g.cm^3$. Blood volumes remaining in the pipette tips were determined by weighing each tip before and after each pipetting. In addition, whole blood volumes of 500 ul were pipetted using an Eppendorf repeater pipettor.

c. Effect of Haemolyzing Agent - Three haemolyzing solutions were investigated to examine the effect of acidity on CO release. These were:

i) 0.5 ml of a 2:1 mixture of 10% aqueous potassium ferricyanide solution and 10% aqueous Triton X-100 solution;

ii) 0.5 ml of a 1:1 mixture of 20% aqueous potassium ferricyanide solution and 1% Triton X-100 in 2N hydrochloric acid; and

iii) 0.5 ml of a 1:1:1 mixture of 20% aqueous potassium ferricyanide solution, 1% Triton X-100 in 2N hydrochloric acid and 50% aqueous hydrochloric acid.

A sample of fresh blood was taken from a subject who regularly used cigarettes, (smoker's blood), and 5 analyses were carried out using each of the above haemolyzing solutions.

In order to assess the effect of injecting the haemolyzing solution through the septum seal of the reaction vial, a further experiment was carried out by preparing standards using 0.5 ml of pH 8.2 buffer solution and 10 ul of carbon monoxide (CO) as in the normal method (Annex A) but with 0.5 ml of the haemolyzing solution added before the vial is sealed and the CO added. Five determinations were carried out using GC and compared to a parallel set of five standards prepared normally with the haemolyzing solution injected through the septa after adding the CO.

d. Effect of Time of Reaction - The effect of the difference in time between the addition of haemolyzing agent to blood and injection of a headspace sample to the GC was assessed for time differences (reaction times) of 2 min, 5 min, 10 min, 30 min and 60 min. Samples were run in duplicate using 10 ul CO standards, saturated haemoglobin solution and whole blood.

e. Sample Transfer from Vial to GC Sample Loop - During the transfer of a 1 ml headspace sample from the vial to the GC sample loop, there is a possibility of exchange occurring between the sample gas and the ambient laboratory atmosphere. To estimate any effect, a small valve was fitted between the barrel and needle of the syringe used for sample transfer. With the valve open, the syringe needle was pushed into the sample vial and a headspace sample taken. The valve was closed while the syringe needle was still in the sample vial and the syringe needle was then withdrawn. The syringe needle was then removed, as in the normal method, and the syringe attached to the GC inlet line. The valve was then opened, the sample was flushed through the sample loop and the GC analysis continued normally. Ten samples of 10 ul CO standards were analysed with the valve in place, and compared to 10 analysed with no valve being used.

f. Effect of Catalyst Temperature - The temperature of the nickel catalyst used to convent CO to CH_4 in the GC analysis was varied over the range of 250°C to 550°C in 50°C increments. Five determinations of CO were made at each temperature using a 500 ppm CO in air primary standard gas mixture.

g. Variability of GC Analysis Step - Primary standard gas mixtures containing 10, 25, 100, and 500 ppm CO in air or nitrogen were used to establish the variability of injection and linearity of response to the GC analysis of CO. Ten injections of each gas mixture were made to the GC.

h. Overall Method Variation - Samples of blood from a non-smoker, light smoker and heavy smoker were obtained. Ten replicate samples of each were analysed to obtain a measure of overall variation in the method.

2. Determination of Total Haemoglobin Concentration

An integral part of the determination of COHb in blood is the measurement of total haemoglobin concentration. The methodology of this determination is outlined in Annex A and entails reaction of an aliquot of the blood with an oxidizing and cyanating reagent, usually referred to as Drabkin's solution. Haemoglobin species in the blood sample are converted to cyanmethaemoglobin (CNMetHb) and its absorbance is determined at 540 nm. This determination is carried out to give a direct read-out of %Hb using a haemophotometer (Fisher Model 74D).

As previously discussed, it has been reported (39) that the rate of reaction of COHb with Drabkin's solution is significantly less than the rates of reaction of O_2Hb or MetHb. Thus, an error can be introduced into the determination of total Hb if the currently recommended reaction time of 10 minutes is used. It has also been reported (39) that higher concentrations of ferricyanide in the reagent will increase the rate of reaction such that 10 minute reaction times can be used at high COHb concentrations. These observations were investigated to determine the extent of possible error and to verify the effect of increased ferricyanide concentration.

a. Effect of Reaction Time - Three blood samples were saturated with CO and dilutions were prepared at nominal concentrations of 100%, 50%, 10% and 0% COHb. Aliquots of 0.020 ml of each dilution were pipetted into cuvette tubes containing 5 ml of Drabkin's solution. Ten replicates of each dilution (100%, 50%, 10% and 0% COHb) for each sample were prepared in this way. Absorbance was read in the haemophotometer at 10 min and 30 min after addition and then at 30 min intervals up to a total time of 240 min.

b. Effect of Reagent - A blood sample was saturated with CO and dilutions at 100%, 50%, 10% and 0% nominal COHb concentration were prepared. Three reagent solutions were prepared as follows:

i) Drabkins solution containing (per litre):

Potassium ferricyanide	200 mg
Potassium cyanide	50 mg
Sodium bicarbonate	1000 mg

ii) Solution of Van Kampen and Zijlstra (38) (per litre):

Potassium ferricyanide	200 mg
Potassium cyanide	50 mg
Potassium dihydrogen phosphate	140 mg

iii) Solution of Taylor and Miller (39) (per litre):

Potassium ferricyanide	1000 mg
Potassium cyanide	50 mg
Potassium dihydrogen phosphate	140 mg

0.020 ml of each prepared blood dilution were pipetted into 5 ml of each of the three solutions above. Five replicates were run for each dilution/reagent combination. Absorbance was read in the haemophotometer at 10 min, 30 min, 60 min and then every 60 min to a total time of 240 min.

3. Investigation of Standardization Procedures

The most important part of the analysis procedure involves the preparation of a standard against which an unknown sample concentration is measured. Procedures examined for standard preparation included an assessment of the current procedure in which 10 ul of pure CO are injected into a sealed reaction vial containing 0.5 ml of pH 8.2 buffer, a modification of this procedure in which the whole headspace of a reaction vial is flushed with a primary standard gas mixture of 500 ppm CO in nitrogen, and procedures using whole blood saturated with CO and haemoglobin extracts saturated with CO. Methods for saturating whole blood and haemoglobin solutions with CO were investigated to find the most efficient method. The procedures used are outlined below.

a. Variation of 10 ul CO Standard - 10 ul CO standards were run for every COHb analysis carried out during the study. The area counts from the integrator-recorder were analysed statistically to obtain a measure of long term variation. Within day variation was assessed by running 10 standards on each of five different days.

b. Use of Other Gas Standards - To assess the effectiveness of using higher concentrations of CO gas in the vial, analyses were run in which 25 ul of CO had been injected into 10 vials and 50 ul of CO had been injected into 10 vials. In addition, 25 ul CO standards were run in parallel with 10 ul CO standards over a period of one month in order to assess long term variability.

The syringe injection of 10 ul of CO into the vial headspace is a possible source of error. To investigate an alternative to this procedure, a series of 10 samples were prepared for which a 500 ppm CO in nitrogen gas mixture was flushed

through the headspace of each vial. These were compared directly to a set of ten 10 ul CO standards. Comparisons were run daily over a two-week period to assess day-to-day variation.

c. Saturation Experiments - Saturation experiments were carried out using whole blood and haemoglobin extracts. The haemoglobin extracts were prepared from whole blood using the method of Collison et al. (6). Using this method, 10 ml of fresh heparinized whole blood were diluted with 5 volumes of 0.9% w/v NaCl solution and the red cells recovered by centrifugation. The cells were resuspended in the same volume of 0.9% NaCl solution and recentrifuged. For each volume of packed cells, 0.1 volumes of 10% aqueous Triton X-100 solution and 3 volumes of pH 8.2 borate buffer were added. The solution was mixed by inversion, allowed to stand for 5 min for complete haemolysis to occur and centrifuged. The clear haemoglobin extract was used in subsequent experiments. Fresh extract was prepared as needed. Fresh whole blood was obtained from both non-smoking and smoking volunteers as required.

i) Syringe Saturation - Whole blood and haemoglobin solutions were saturated using the method of Collison et al. (6) as follows:

Approximately 15 ml of either whole blood or haemoglobin solution were transferred to a lightly oiled 100 ml glass syringe equipped with a 3-way stopcock. All air was expelled, and approximately 35 ml of pure CO was introduced to the syringe. The syringe was either rotated by hand or placed on a rocking shaker for at least 15 min to effect saturation. The unabsorbed CO was expelled from the syringe, and approximately 50 ml of pure helium introduced. The contents were again mixed for at least 15 min and all gas expelled. The solution was stored anaerobically in the syringe at refrigerator temperature when not being used. Saturated solutions prepared in this way were evaluated in comparison with other saturation procedures by the GC method using 0.1 ml sample aliquots.

ii) Simple Bubbler Method - The first method investigated involved placed a 5 ml aliquot of blood or haemoglobin solution in a test tube, adding 1 drop of n-octanol to suppress foaming, and bubbling pure CO through the solution at an approximate flow rate of 30 to 40 ml/min using a pasteur pipette as the bubbler. The CO gas was first passed through a flask containing distilled water to minimize water loss from the blood sample. CO was

bubbled for 30 min and 1 hour periods, with a 5-min flush with helium after each saturation to remove dissolved CO.

iii) Impinger Method - The second method used a midget impinger into which 10 ml of blood was placed. One drop of n-octanol was again used as an anti-foaming agent, and the CO was saturated with water vapour by passing through two midget impingers containing distilled water. A flow rate of 30 to 40 ml/min was again used and CO bubbled for 30 min and 1 hour periods with a 5-min helium flush to removed dissolved CO.

iv) Heated Impinger Method - The midget impingers were placed in a water bath maintained at 37°C (approximate body temperature) and the sample was saturated according to the protocol described in (iii) above.

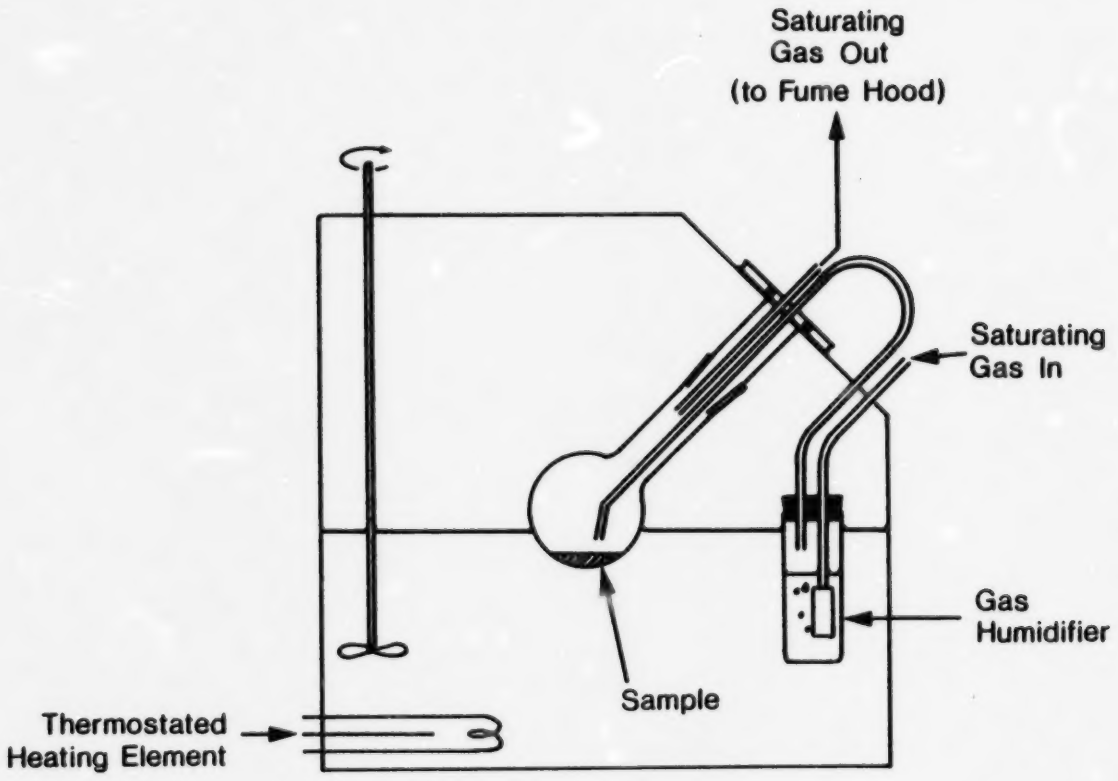
v) Rotating Tonometer Method - A 10 ml aliquot of blood or haemoglobin solution was placed in a 250 ml tonometer flask which was attached to a rotating head as illustrated in Figure 3. The part of the flask containing the blood was immersed in a water bath maintained at 37°C. CO was bubbled through two flasks containing distilled water also maintained at 37°C and then passed over the blood or haemoglobin aliquot in the flask. The flask was rotated causing the sample to be spread in a thin film around the bottom of the flask and thus promote efficient gas exchange across the surface to effect saturation. Three flow rates of CO, 100 ml/min, 200 ml/min and 400 ml/min were investigated to determine the optimum flow for efficient saturation, with a minimum concentration effect on the haemoglobin due to uptake or loss of water vapour from the sample. Saturation times of 30 min and 1 hour were used with a 5-min flush with a 1% CO in helium mixture for each to remove dissolved CO. The CO gas mixture was used as this has been found to remove dissolved CO without removing any bound CO (42). Saturation experiments were carried out using whole blood and haemoglobin extract from whole blood.

vi) Reduction with Dithionite - To assess the influence of methaemoglobin (MeHb) present in the blood or haemoglobin solutions, saturations were also carried out on solutions to which sodium dithionite had been added (approximately 40 mg to 10 ml sample) to reduce any methaemoglobin present to haemoglobin.

vii) Effect of Dilution - Dilutions of saturated blood and saturated haemoglobin solutions were carried out to assess the feasibility of using such dilutions as

FIGURE 3

Diagram of Rotating Tonometer Operation



standards. Mixtures were prepared using a saturated solution and its unsaturated precursor solution to obtain 50%, 25%, 20%, 15%, 10%, 5% and 2% dilutions. After preparation, the solutions were stored anaerobically in oiled 5 ml glass syringes at refrigerator temperature. Analyses were carried out daily over a 10-day period to assess stability of the different dilutions. Two series of dilutions were prepared, one using blood from a light smoking subject, and one from a heavy smoking subject.

viii) Effect of Long-term Storage - Mixtures were prepared using a CO saturated blood solution and its unsaturated precursor solution to obtain 50%, 25%, 20%, 15%, 10%, 5% and 2% dilutions. After preparation, the solutions were stored anaerobically in oiled 5 ml glass syringes at refrigerator temperature. Analyses were carried out immediately, after 1 day, 3 days, 5 days, 10 days, and every 10 days until the sample was depleted. This was sufficient to give 60 to 80 days of analyses.

4. Comparison with Other Analysis Techniques

As part of a larger study, human subjects were dosed with CO in air mixtures. Blood samples were taken before and after each dosing with CO and analysed using the GC method and CO-oximetry. These results were compared to estimates of COHb concentration made from CO concentration measured in concurrent samples of alveolar air using an electrochemical CO analyzer (Ecolyzer Series 2000 Analyzer).

RESULTS AND DISCUSSION

1. Precision and Accuracy of the Method

Variation within the analytical method can be ascribed to several sources. Major sources of determinant error are:

- variation in vial volume.
- variation in the volume of blood delivered into the vial.
- variation in the volume of gas standard measured.
- variation in the amount of CO liberated by the releasing agent.
- variation in time between addition of CO releasing agent to the blood sample and analysis by GC.
- variation in volume of headspace gas delivered to the GC.
- variation in ambient temperature and pressure.

Indeterminant and other sources of error would be largely due to:

- operator error.
- small changes in GC column temperature and carrier gas flow rate.
- efficiency of the nickel catalyst in converting CO to methane prior to detection.
- CO displacement from the sample during flushing of the vial with helium (variation depends on COHb concentration of the sample).
- CO loss through the vial septum during injection of the releasing agent.
- gas exchange with the atmosphere between the time when a headspace sample is drawn into the sampling syringe and its attachment to the GC inlet system.
- CO loss during mixing of the blood sample prior to taking an aliquot for analysis.
- absorption of some gaseous CO into the liquid remaining in the vial after CO release.

Results of experiments to quantify these sources of variation are presented below:

a. Vial Volume - Vial volume was found to be 23.3 $\pm$ 0.3 ml for one batch of vial and 21.8 $\pm$ 0.1 ml on a second batch of vials. Only two batches of vials were used during the course of this study, however, the 1.5 ml variation between them indicates that volume measurements should be made on each batch of vials used for this analysis. Within batch variation, however, was found to be quite small with coefficients of variation (CV) of 1.3% and 0.6% for respective batches.

b. Blood Volume - Using Eppendorff single delivery pipettes, three different operators found that a 500 ul delivery was actually only 470.5 ± 3.4 ul, 479.1 ± 1.8 ul and 471.5 ± 4.5 ul, respectively, with the average being 473.5 ± 4.7 ul. Two operators found a 100 ul delivery to be 94.7 ± 0.7 ul and 91.9 ± 0.9 ul respectively, with the average being 93.3 ± 1.7. Thus, a considerable error can arise in measuring blood volume and this error is somewhat dependent on the operator carrying out the measurement. A separate experiment determined that the amount of blood left coated on the inside of the pipette tip after delivery was sufficient, within experimental error (1% CV), to account for the volume difference. This problem was solved by using a repeating delivery pipette (Eppendorff Repettor). This type of pipette takes up a larger volume of sample into a reservoir and dispenses aliquots whose volume is determined by the reservoir size and the pipette setting. Using this device, 500 ul deliveries were found to be 498.9 ± 2.0 ul and 100 ul deliveries were found to be 98.6 ± 0.6 ul. The first and last aliquots using this pipette are discarded to avoid volume measurement errors due to blood films in the pipette tip.

c. Effect of Haemolyzing Agent - The effect of haemolysing (releasing) agent was examined using different concentrations and acidities to release CO from a sample of smoker's blood. The results are summarized in Table 1. The least acidic reagent released the lowest amount of CO from the blood, even after standing for more than 30 min. The "normal" agent (2) and a more acidic agent (3) released a significantly higher concentration of CO into the vial headspace. However, the difference in %COHb determined between the normal agent and the more acidic agent is not significant, which suggests that the normal agent is sufficiently acidic to release all available CO from the blood sample.

d. Loss of CO Through Vial Septum Puncture

The possibility of loss of CO through the vial septum during injection of the haemolysing agent was investigated by running 10 ul CO standards to which haemolysing agent was added before and after the vial was sealed. Average peak area for addition of haemolysing agent before sealing the vial was 13860 ± 310 counts, whereas average peak area for addition of haemolysing agent after sealing the vial was 13610 ± 290 counts. A t-test between means did not show a significant difference between these two values. Thus, it can be concluded that no loss of CO occurs through the septum vial after puncturing to add haemolysing agent.

TABLE 1

Effect of Haemolysing Agent on Determined % COHb Level
(0.5 ml sample used in all cases)

	Haemolysing Agent	% COHb	% CV of Determination
1.	0.5 ml of a 2:1 mixture of 10% potassium ferricyanide solution & 10% aqueous Triton X-100 solution	10.9 ± 0.7	6.3
2.	0.5 ml of a 1:1 mixture of 20% potassium ferricyanide solution & a 10% solution of Triton X-100 in 2N hydrochloric acid	12.5 ± 0.2	1.7
3.	0.5 ml of a 1:1:1 mixture of 20% potassium ferricyanide solution, 10% Triton X-100 in 2N hydrochloric acid & 6N hydrochloric acid solution	12.4 ± 0.2	1.2

e. Effect of Time of Reaction - The effect of time of reaction between addition of the releasing agent to the vial and injection to GC, is summarized in Table 2. Inspection of the results indicates that while considerable variation is seen (CVs of around 10%), no trend showing an effect of reaction time is observed.

TABLE 2

Effect of Reaction Time on GC Peak Area and Determined % COHb Levels

Reaction Time (min)	Peak Areas			% COHb	
	10 ul CO	Whole Blood (Smokers)	CO Sat Hb Solution	Whole Blood[a] (Smoker)	CO Sat[b] Hb Soln
2	13290± 480	6800±320	7960±110	4.1±0.2	86.5±1.8
5	12640±2710	7140±230	9130±140	4.3±0.9	99.1±2.2
10	14160± 950	7090±200	8820±850	4.2±0.3	95.7±9.3
30	14900± 100	6850±520	8130±180	4.1±0.3	88.3±2.4
60	12800± 320	7000±140	9590±370	4.2±0.1	104.1±4.3
Overall Mean	13280±1340	6980±380	8720±830	4.2±0.5	94.7±9.1
%CV	10.1	5.4	9.5	11.5	9.6

[a] Calculated using standard peak are of 13280, 16.6% Hb, 741 mmHg pressure and 299 K ambient temperature

[b] Calculated using standard peak area of 8300, 3.7% Hb, 745 mmHg pressure and 297 K ambient temperature

f. Sample Transfer from Vial to GC Sample Loop - The possibility of exchange of syringe contents with the ambient atmosphere during transfer of a headspace sample from the vial to the GC inlet was investigated using a small valve to close off the syringe during the transfer operation. With the valve in place, a series of injections of a 10 ul CO standard resulted in an average peak area of 12740 ± 670 counts, compared to an average peak area of 13130 ± 240 counts with no valve. A statistical t-test did not show these two results to be from different populations at the 95% confidence level. This indicates that gas exchange with the ambient atmosphere does not occur at a significant level within the experimental error of the method.

g. Effect of Catalyst Temperature - The nickel catalyst used to convert CO to CH_4 in the GC is normally kept at a temperature of 400°C. To determine if variations in catalyst temperature have an effect on peak area measured, the temperature was varied between 250°C and 500°C in 50°C increments while observing the response to 100 ul injections of a 500 ppm CO in air primary standard mixture. The results are illustrated in Figure 4 and show that response is quite even in the 375-500°C range. This indicates that normal temperature fluctuations of ± 10°C around the 400°C set-point will not affect observed peak areas.

h. Variability of Gas Analysis Step - Variation in the volume of pure CO gas used to standardize the method was measured for 10 ul, 25 ul and 50 ul volumes of CO injected into the vial headspace. Table 3 summarizes the results of this experiment.

TABLE 3

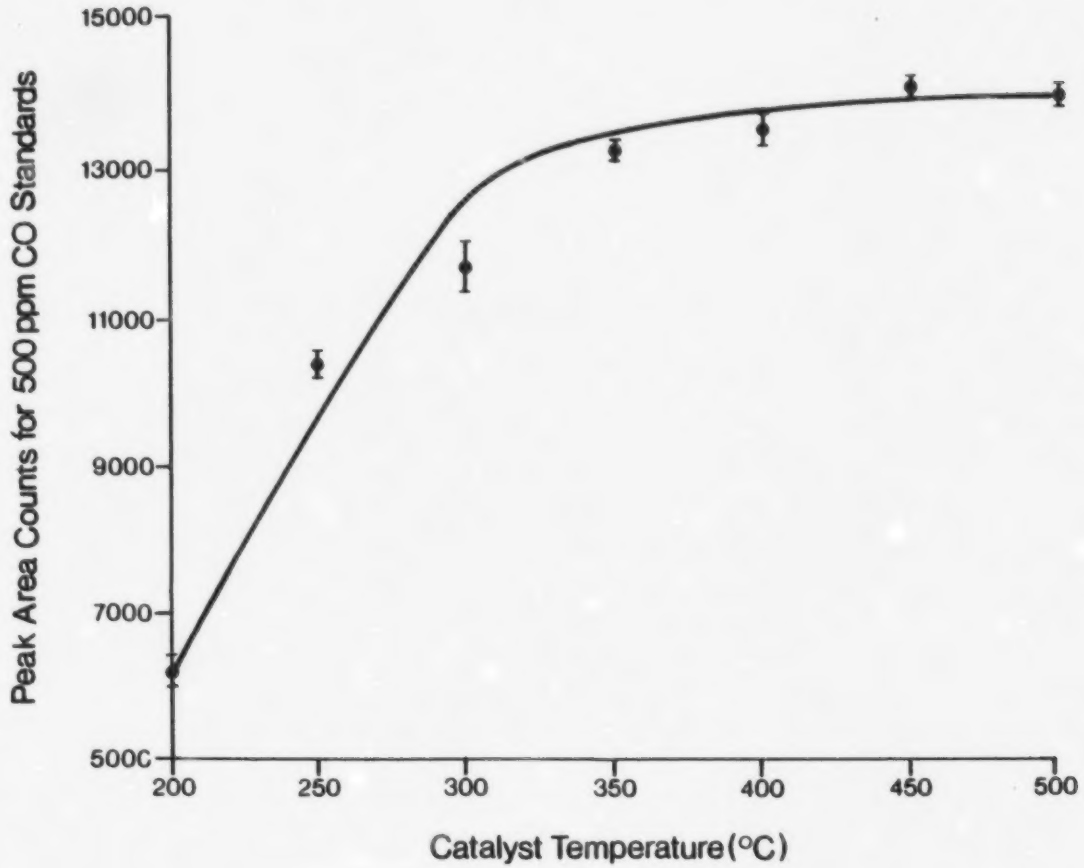
Variation of 99.5% CO Injections

Volume of CO Injected	Average Peak Area Counts	Standard Deviation	C.V.%
10 ul	9245	382	4.1
25 ul	23685	203	0.9
50 ul	46508	2064	4.4

Two gas syringes, one of 25 ul capacity and one 100 ul capacity, were used; the 25 ul syringe to measure 10 ul and 25 ul and the 100 ul syringe to measure 50 ul. The relatively high % C.V. for the 50 ul injections compared to the 10 ul and 25 ul results are explained by the lower precision of using a

FIGURE 4

Effect of Catalyst Temperature on Observed Peak Area



100 ul syringe. The variation shown in Table 3 also i ides variation in vial volume and variation of injection the GC. Variation of injection to the GC was tested by inj ting primary standard gas mixtures of different concentrati s of CO in nitrogen or air into the GC. Four injections for each concentration were made and averaged and the results are presented in Figure 5 and summarized in Table 4. A straight line plot is obtained with an r^2 value of 1.00. The decrease in % C.V. with increase in CO concentration is expected because of lower variability at larger peak areas. The % C.V. for 500 ppm CO concentrations is 1.1%. This concentration is approximately equivalent to the concentration in the vial headspace containing 10 ul of pure CO (450 ppm). Thus, taking account of approximately 1.0% C.V. for vial volume variation and 1.1% C.V. for GC injection variation, the within day variability of the 10 ul CO standard is 3.8% C.V. However, the peak areas in Table 3 and 4 cannot be directly compared because a significant variation of peak area can occur over time periods longer than 4 to 5 days. This point will be discussed more fully below.

TABLE 4

Variation of Injection to GC Using Standard Gas Mixtures[a]

CO Concentration (ppm)	Average Peak[c] Area	Cumulative[b] % CV	Equivalent[d] % COHb
10.5	330	17.5	0.21
25	760	5.7	0.49
100	3050	2.7	2.0
500	15350	1.1	9.8

[a] Matheson Primary Standards reported concentration ± 1.0%

[b] Obtained from four sets of data where

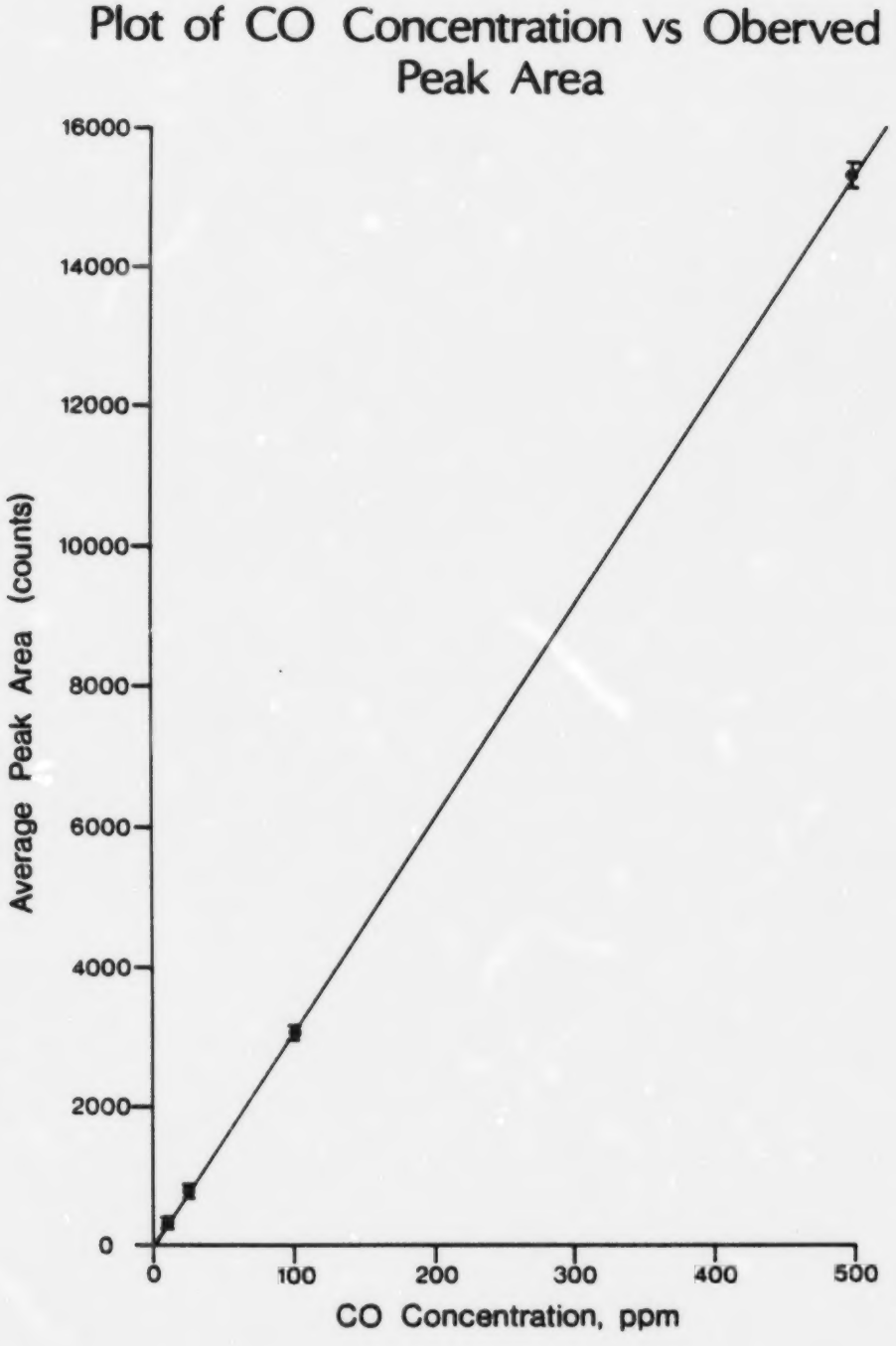
$$\text{Cum \%CV} = \sqrt{(\%CV)^2 + (\%CV)^2 + (\%CV)^2 + (\%CV)^2}$$

[c] Normalized to 500 ppm mixture GC attenuation level

[d] Assuming 0.5 ml blood sample, 15% Hb, and 23.1 ml vial volume at 745 mmHg and 298 K ambient temperature and pressure

i. Overall Method Variation - Overall method variation was assessed by determining COHb levels on whole blood samples taken from non-smoking, smoking and CO-dosed subjects. The results are summarized in Table 5. COHb levels ranged from 0.64% for non-smoker's blood to 18.1% in blood after dosing with CO. The coefficient of variation ranged from 1.1% to

FIGURE 5


Plot of CO Concentration vs Oberved Peak Area
Average Peak Area (counts)
16000
14000
12000
10000
8000
6000
4000
2000
0
0
100
200
300
400
500
CO Concentration, ppm

12.3% with an average of 6.2%. The previously estimated variation of the 10 ul CO standard of 4.1% CV suggests that indeterminant errors in the method account for a variation of 4.7% to give this combined CV of 6.2%.

TABLE 5

Variation of %COHb at Various Concentrations

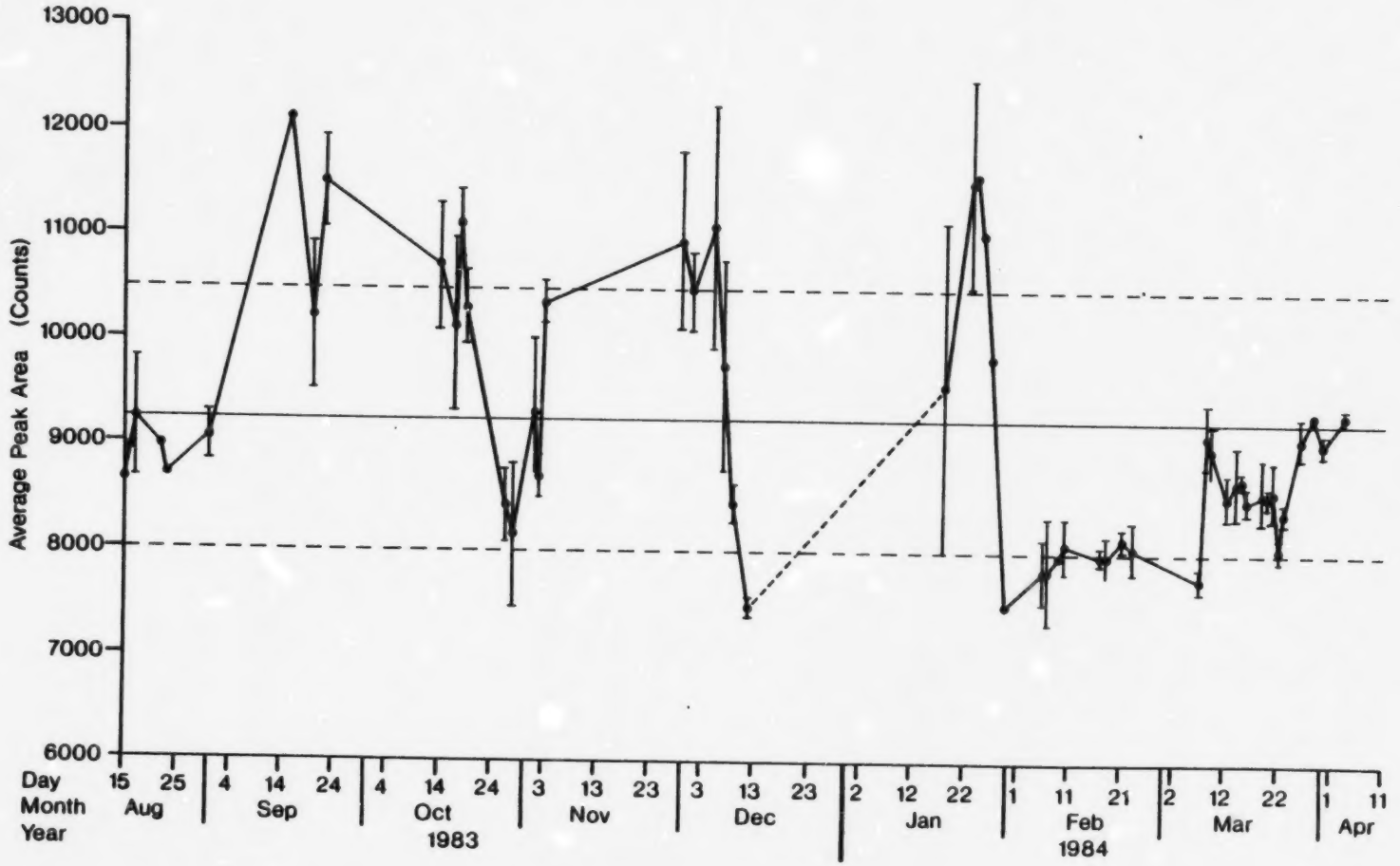
Subject	%COHb	% CV
non-smoker	0.64 ± 0.02	2.8
non-smoker	0.64 ± 0.08	12.2
non-smoker	0.76 ± 0.03	4.0
light smoker	1.8 ± 0.1	2.9
light smoker	2.4 ± 0.1	4.9
light smoker	2.3 ± 0.2	7.6
moderate smoker	4.8 ± 0.4	8.9
moderate smoker	5.7 ± 0.1	1.0
heavy smoker	8.6 ± 0.1	1.1
heavy smoker	9.5 ± 0.7	6.9
CO-dosed[a]	14.1 ± 1.7	12.3
CO-dosed[a]	17.3 ± 1.0	5.7
CO-dosed[a]	18.1 ± 1.9	10.6
	Average % CV	6.2

[a] Dosed with 6000 ppm.min under various protocols. %COHb reported is final level reached

One of the major sources of variation in both the standard determination and COHb determination is fluctuation in chromatographic conditions. Figure 6 illustrates the variation of peak area with time for the 10 ul standard over an 8-month period. The average peak area during this period was 9250 counts with a standard deviation of 1250. All data reported in Figure 6 are corrected to STP (760 mmHg, 273 K). Within day standard deviations are shown as bars on the data points in Figure 6. Large changes in peak area can be largely attributed to changes in flow rate. Adjustment of the flow rate accounts for the large shift on Sept 15, 1983, with a gradual erosion of peak area caused by a gradual decrease in flow rate and buildup of impurities on the GC column. Other sharp changes can be associated either with flow rate adjustments or column bake-out. These results show the importance of determining a standard peak area on the same day as samples are analyzed. GC peak areas seldom remain constant within a 5% variation range for more than 5 to 7 days using the current experimental set-up without adjustment of flow rate and regular bake-out of the columns to remove adsorbed water, carbon dioxide and other impurities.

FIGURE 6

10µl CO Standard daily average peak area counts.



Accuracy of the method was checked by comparison with spectrophotometric procedures. The COHb content of haemoglobin solutions was checked by determining absorbance at a wavelength of 540 nm using the procedure of Collison et al. (6). The results of four determinations are listed in Table 6.

TABLE 6

Comparison of % COHb Levels in Haemoglobin Extracts Using GC and UV-Visible Spectrophotometry

% COHb		
GC	Spectrophotometry[a]	Difference
86.1	92.3	+6.2
81.0	88.4	+7.4
79.3	87.5	+8.2
96.5	103.9	+7.4
	Average	+7.3

[a] Determined by method of Rodkey et al (10) using a Beckmann Model ACTA MV-1 UV-Visible Spectrophotometer

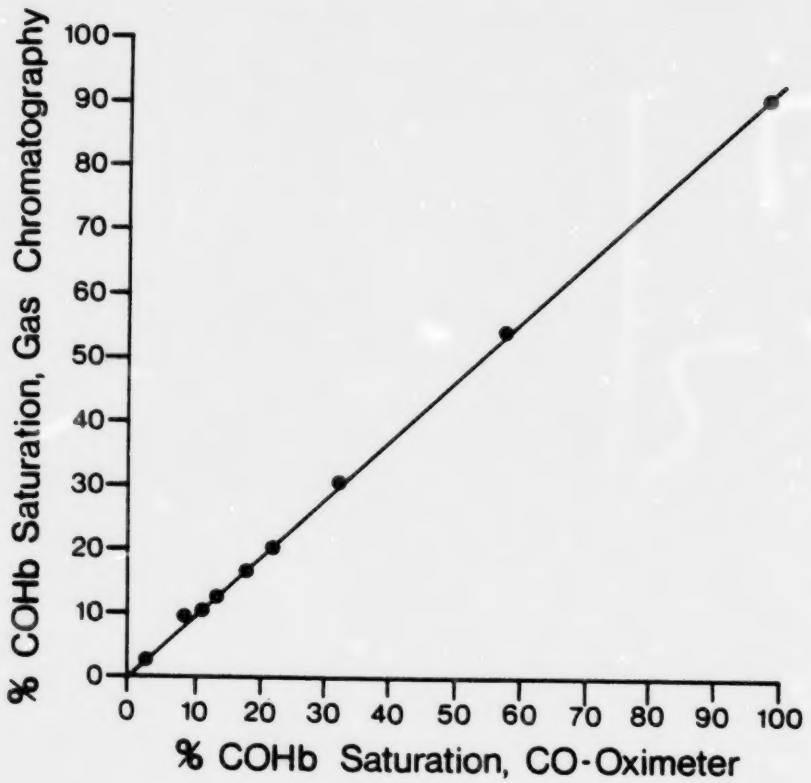
It can be seen that the current GC method gives results that are approximately 7% lower than the spectrophotometric method. To further define this relationship, a series of solutions containing a range of from 2% to 100% COHb saturation were prepared and analysed both by GC and by CO-oximetry using a Corning CO-oximeter. The results are illustrated in Figure 7. Data points are averages of 4 determinations carried out over a 7-day period. Excellent co-linearity between the two methods was obtained with a regression of:

$$(COHb_{(GC)}) = 0.924\ (COHb_{(CO\text{-}ox)}) + 0.100$$

and an r^2 of 1.00. However, the GC method gave results that were 7.5% lower than the CO-oximeter reading over the entire range of the experiments. Possible interference with the GC method due to high levels of methaemoglobin were ruled out as such levels determined by CO-oximetry were found to account for only approximately 1% of total haemoglobin in the samples measured. As these experiments were carried out using Eppendorff single delivery pipettes to measure blood volumes, most of this bias (5-6%) is due to the tip coating effect described previously. The remaining bias is probably due to error bias in measuring total Hb, as described in the next section.

FIGURE 7

Comparison of % COHb Determination between GC and CO-Oximeter Analysis Methods



2. Determination of Total Haemoglobin Concentration

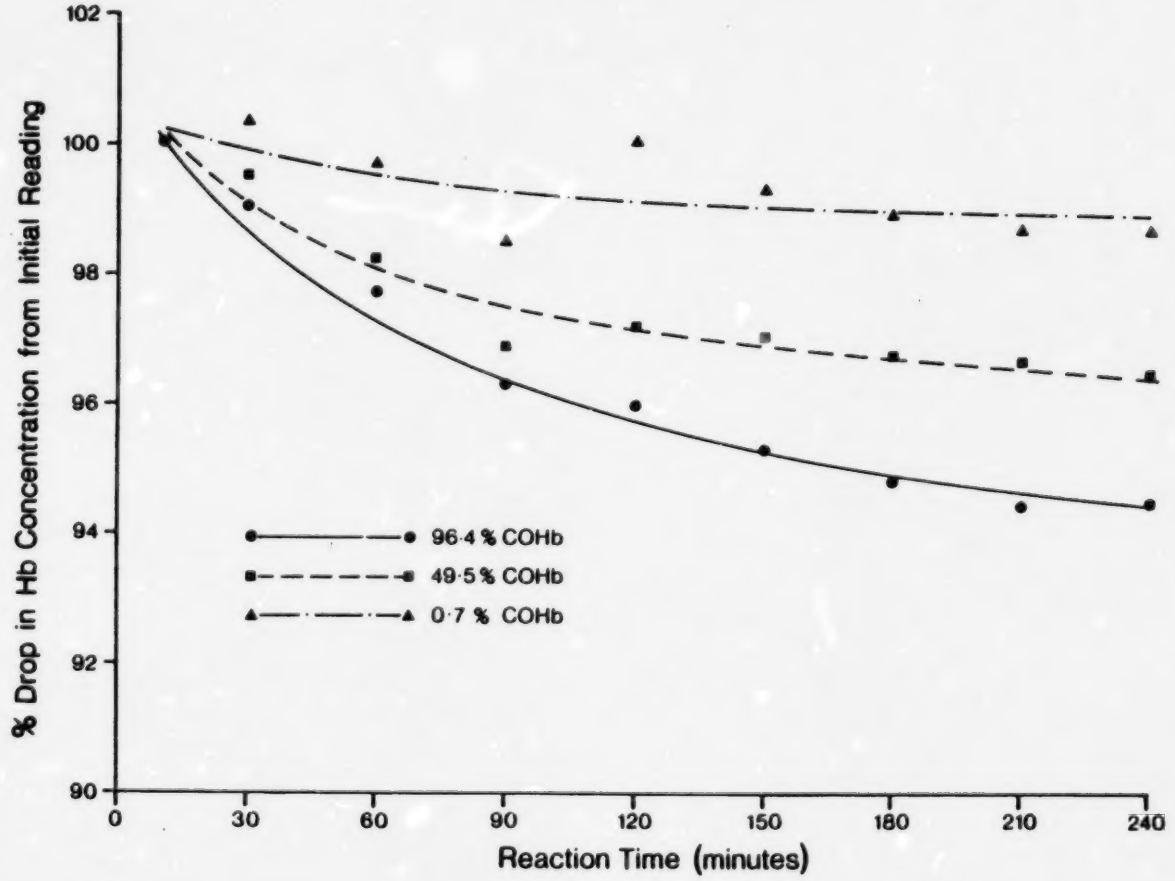
Experiments were carried out to determine the effect of COHb concentration on total Hb concentration and also the effect of reagent composition on the required reaction time for total conversion of Hb species in blood to CNMetHb at various concentrations of COHb. The results are presented below:

a. Effect of Reaction Time - Aliquots of blood containing nominally 100%, 50% and 0% COHb from three different blood samples were used to estimate the effect of reaction time. Ten replicates were run for each COHb concentration for each sample at each measurement interval during the experiment. To eliminate the effect of different Hb concentrations between different blood samples, the percent decrease in Hb concentration with time for each replicate was calculated using the value recorded at the 10 minute reaction time as 100%. A decrease in the observed Hb concentration with more complete reaction to CNMetHb is expected, as the molar absorptivities of both HbO_2 and COHb are approximately 32% greater than the molar absorptivity of CNMetHb at a wavelength of 540 nm (39). The results are presented in Figure 8, where each point represents the average of all replicates for that reaction time. All dilutions show a decrease over time indicating that the conversion to CNMetHb is slow using Drabkin's solution even at low levels of COHb. However, it can be seen that at higher COHb concentrations, the use of a 10 minute reaction time can introduce errors into the determination of Hb concentration of approximately 3.5% at 50% COHb saturation and 5.5% at 100% COHb saturation. The curves shown are best fitted statistically with power curve relationships. However, the curve for 0% (0.7%) COHb has an r^2 of 0.48, indicating poor fit, and the curves for 50% (49.5%) and 100% (96.4%) COHb, although having r^2 values above 0.93, are not well fitted to the experimental data in the 10 to 90 minute range. This indicates that the relationship is more complex than a simple power curve fit, perhaps because of the two-step reaction process involved.

b. Effect of Reagent - In order to ensure that all Hb species, including COHb, in a blood sample are converted to CNMetHb in the determination of total Hb concentration, the effect of different reagents were investigated. Apart from the standard Drabkin's solution, a solution recommended by the International Committee on Standardization in Haemotology (38), and a modified version of this solution in which the potassium ferricyanide concentration was raised from 200 mg/L to 1000 mg/L (39), were investigated. The results of this experiment are presented in Table 7. From inspection of the Table, it can be seen that the reaction time retardation

FIGURE 8

% Decrease in Hb Concentration from Initial Reading as a Function of Reaction Time



effect is small at COHb concentrations below 20%. The Drabkin's solution and Van Kampen and Zijstra solution both show similar retarded conversion to CNMetHb with time at higher COHb concentrations. For the Taylor and Miller solution, the effect of COHb retarding conversion is only observed for the 100% COHb sample and the reaction is complete in 60 minutes as compared to 180-240 minutes with the other reagent solutions. The increased level of potassium ferricyanide helps give faster analysis times for total Hb determination by increasing the oxidation rate of COHb to MetHb in the first step of conversion to CNMetHb and thus, this solution is recommended for future analyses.

TABLE 7

Effect of Reagent in the Determination of Total Haemoglobin Concentration at Various CO Saturation Levels

% COHb	Time of Reaction (min)					
	10	30	60	120	180	240
Using Drabkin's Solution						
100	16.1	16.0	15.8	15.6	15.4	15.4
50	15.3	15.3	15.1	15.0	14.9	14.9
20	14.8	14.8	14.6	14.7	14.6	14.6
10	14.7	14.7	14.6	14.6	14.6	14.6
0	14.7	14.7	14.6	14.6	14.6	14.6
Using Van Kampen and Zijlstra Solution (38)						
100	16.5	16.3	15.9	15.7	15.6	15.6
50	15.8	15.7	15.4	15.4	15.3	15.3
20	15.3	15.3	15.3	15.2	15.2	15.2
10	15.1	15.2	15.2	15.1	15.1	15.1
0	15.0	15.1	15.0	15.0	15.0	15.0
Using Taylor and Miller Solution (39)						
100	15.7	15.6	15.3	15.3	15.3	15.3
50	15.3	15.3	15.2	15.2	15.2	15.2
20	14.9	14.9	14.9	14.8	14.8	14.8
10	14.9	14.9	14.9	14.9	14.9	14.9
0	14.8	14.9	14.8	14.8	14.8	14.9

3. Investigation of Standardization Procedure

The long term variability of the 10 ul CO standard, as previously mentioned, was found to be 13.5% CV from an average peak area of 9250 counts. However, within day variability over the same period averaged a 4.4% CV, with a range of from 0.5% CV to 17.9% CV. Comparison with a 25 ul standard over a 1-month period showed that the 25 ul standard had the same 13.5% CV over a long period, but that within day variation was 2.6% CV.

As an alternative method, a primary standard gas mixture of 500 ppm CO in nitrogen was used to flush the vial headspace before GC analysis. The results of sets of 10 analyses run over several days are compared to parallel sets of 10 ul CO standards in Table 8.

TABLE 8

Comparison of CO Standards
(all area counts corrected to STP)

Set No.	Average Peak Area Counts: 10 ul CO[a]	500 ppm CO[b]
1	8750±580 (6.6%)[c]	9750±150 (1.5%)
2	9050±310 (2.4%)	9770±160 (1.6%)
3	8480±210 (2.5%)	9420±100 (1.1%)
4	8590±440 (5.1%)	10160±140 (1.4%)
5	8510±440 (5.2%)	9380± 60 (0.6%)
6	8780±310 (3.5%)	9990±270 (2.7%)
7	10000±300 (3.0%)	9790±210 (2.1%)
Overall Average	8890±600	9740±300
Overall % CV	6.7	3.1

[a] 10 ul CO injected to headspace, equivalent to 450 ppm standard; average of 10 determinations
[b] Vial headspace flushed with 500 ppm CO in nitrogen for at least 10 sec; average of 10 determinations
[c] Figures in parentheses are %CV values

It can be seen that the 500 ppm CO mixture has much lower within day variability than the 10 ul CO standard and has less than half of the variability of the 10 ul CO standard even when averaged over several days.

Blood and haemoglobin solutions saturated with carbon monoxide have traditionally been used as standards for the determination of %COHb

levels in blood. Several methods of saturating whole blood and haemoglobin solution have been investigated, as outlined in the Methodology section. The results of these investigations are summarized in Table 9. It can be seen from these results that the use of an impinger or tonometer gives the highest levels of saturation with whole blood being more easily saturated than haemoglobin solutions. However, one tonometer experiment carried out using haemolysed blood achieved a 94.5% COHb saturation. Haemolysed blood is easier to use as a standard because errors due to red cell separation are eliminated.

TABLE 9

Degree of Saturation of Haemoglobin by Different Methods

Method	Type of Solution	Degree of Saturation (%COHb)
Syringe	Hb solution	88.1 ± 10.5
	Whole blood	79.5 ± 8.7
	Purified Hb solution	88.1 ± 3.3
Bubbler		
1. Pipette	Hb solution	78.9 ± 5.8
	Whole blood	84.5 ± 8.3
2. Impinger	Hb solution	94.9 ± 1.7
	Whole blood	96.3 ± 5.1
3. Impinger in 37°C water bath	Hb solution	91.4 ± 4.1
	Whole blood	93.7 ± 8.0
Heated Tonometer	Whole blood	92.3 ± 3.0

Changes in flow-rate using the tonometer had significant effect on degree of saturation. Using a 100 cm^3/min flow-rate of CO gas, haemodilution occurred with the blood Hb concentration falling from 16.1% to 10.8% over a 60-minute period. Resultant CO saturation was 95.4% COHb. Using a 400 cm^3/min flow-rate of CO gas, haemoconcentration occurred with blood Hb concentration rising from 16.1% to 17.5% over a 60 min period, with resultant %COHb of 89.1%. Several determinations using a 200 cm^3/min flow-rate of CO gas gave a slight haemoconcentration effect changing blood Hb concentration by an average of 0.2%, with average COHb concentration of 92.3%. The haemoconcentration effects could be a further reason for the relatively low level of saturation measured. No significant differences in degree of saturation achieved were observed between saturations carried out for 30 min or 60 min. Washing with helium or 1% CO in helium for approximately 5 to 10 min proved adequate for removing dissolved CO.

The use of sodium dithionite to reduce any methaemoglobin present to haemoglobin for subsequent reaction with CO did not produce significantly higher levels of saturation. Solutions containing dithionite gave off sulphur dioxide gas on standing and probably some sulphaemoglobin was formed. Saturations carried out using dithionite were found to yield 95.8 ± 10.0% COHb for haemoglobin solutions and 97.3 ± 4.0% COHb for whole blood compared to saturations of 94.9 ± 1.7% COHb for haemoglobin solutions and 96.3 ± 5.1% for whole blood when no dithionite was added. The differences for the two types of samples are not statistically significant. It was subsequently found by CO-oximetry that methaemoglobin levels in fresh whole blood are normally at a level below 1%.

Dilutions of saturated whole blood were made to test reproducibility at different concentrations and also to investigate the use of such solutions as standard materials. The results of two series of dilutions prepared using blood from a light smoker (2.3% COHb presaturation) and blood from a heavy smoker (9.5% COHb presaturation) are presented in Figure 9. This plot shows the measured COHb level for various dilutions compared to the expected COHb level at that dilution calculated using 100% COHb for the fully saturated solutions and 9.5% and 2.3% COHb respectively for the two diluent solutions. The measured COHb levels are close to the expected COHb levels up to 50% dilution, but are approximately 8.5% too low at the 100% saturation level. Up to the 20% COHb saturation level, all dilutions are within 1% COHb of their expected values, and thus could be used as periodic checks on the method. From results presented earlier, the 8.5% difference can be explained by the combined effects of the pipetting technique used (single delivery, results approximately 4.5% too low) and the method of determining total Hb (Drabkin's solution and 10 min reaction time, results approximately 4.5% too low).

Table 10 lists determinations on the two sets of dilutions over a two-week period. It can be seen that no trend to lower values over the period is seen, and the variability observed can probably be ascribed to other factors such as blank variation and variations in haemoglobin content. Thus, whole blood saturations are stable for periods up to 9 days, provided that they are stored anaerobically at refrigerator temperatures.

The effects of long term storage are summarized in Figure 10 for storage time up to 60 days. The 100% COHb solution was quite stable for 8 days then began to deteriorate fairly quickly over the next 10 days, dropping from 94% COHb to 80% COHb. The COHb concentration continued to fall over the next 40 days to around 70% COHb. Similar, but less marked effects can be observed for the 50% and 20% COHb solutions. The 10% COHb solution was essentially unchanged over the two month time period. Also measured, but not shown in Figure 10, were solutions of 25% COHb and 15% COHb and the behaviour of these solutions were intermediate between the 50% and 20% solutions and the 20% and 10% solutions respectively. Solutions containing less than 10% COHb saturation were unchanged during the experiment.

FIGURE 9

Measured vs Expected % COHb levels for Various Dilutions of Saturated Whole Blood

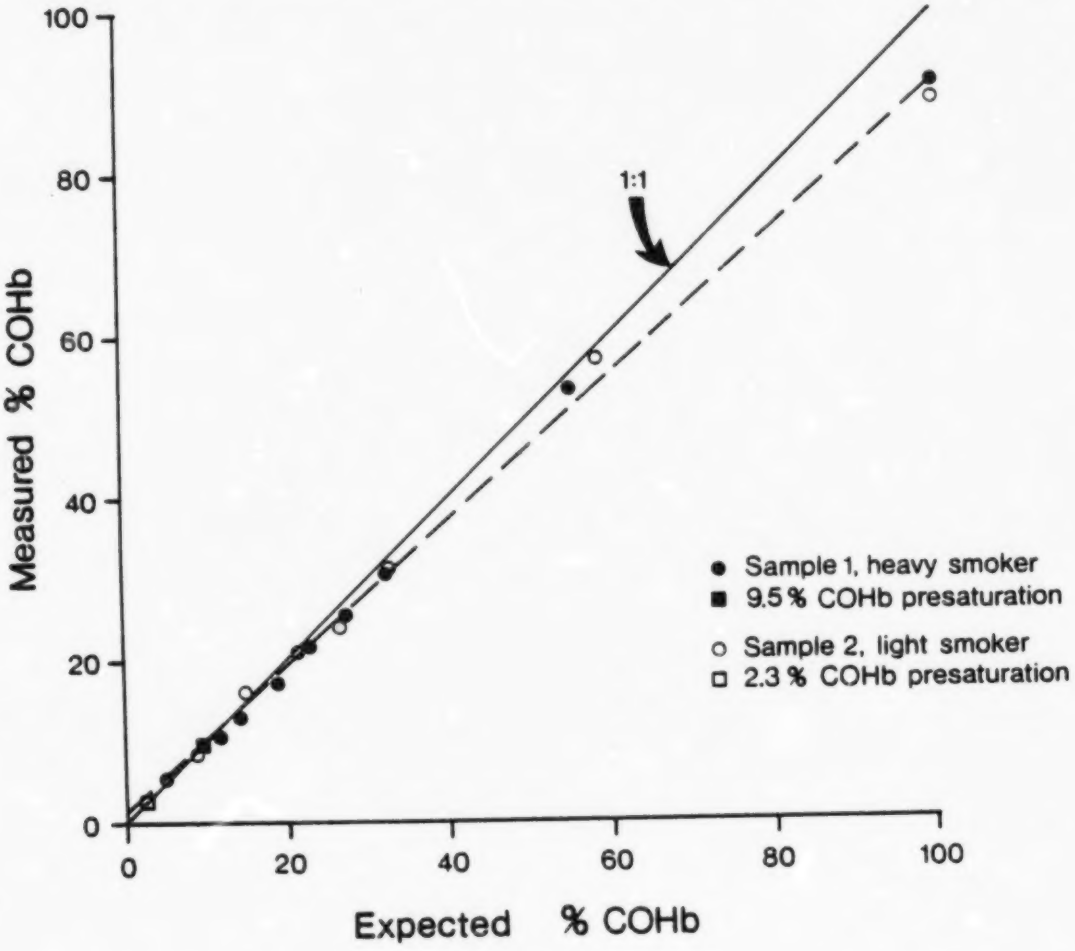


FIGURE 10

Effect of Storage Time on % COHb concentration in Blood

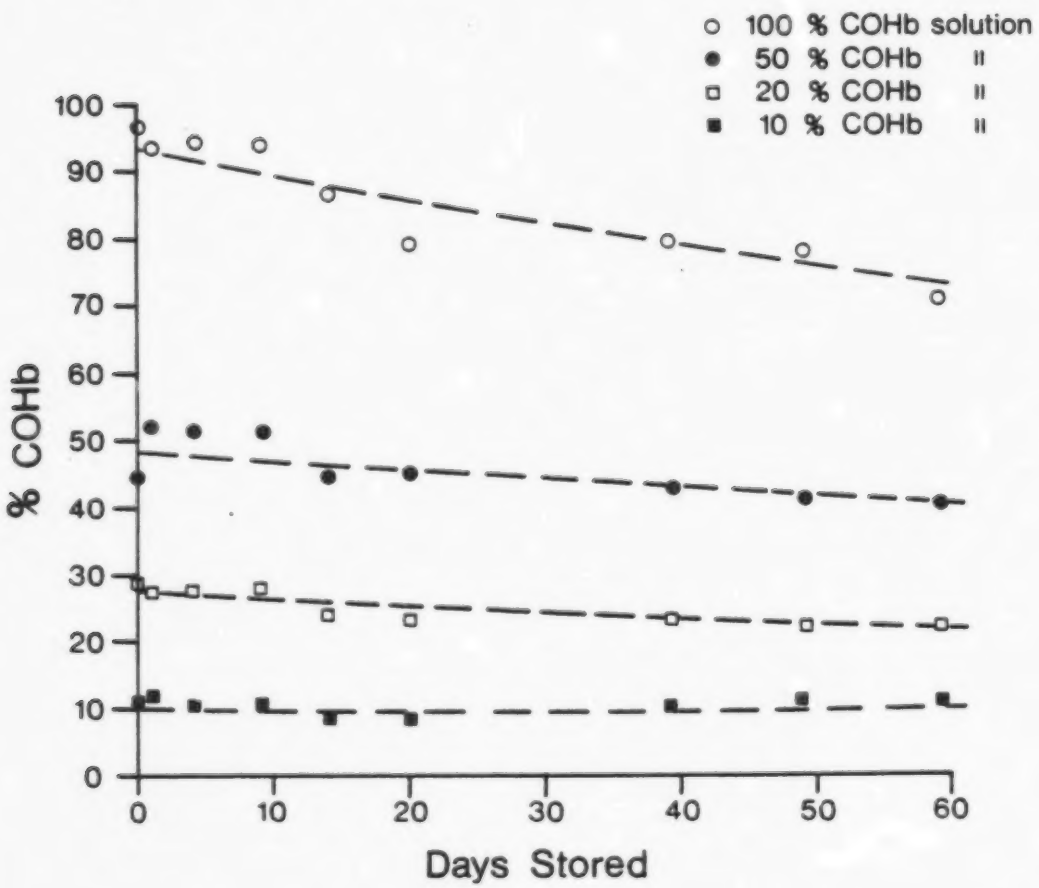


TABLE 10

Stability Over Time of Whole Blood
at Various %COHb Levels

	% COHb at Dilution								
Day	100%	50%	25%	20%	15%	10%	5%	2%	0%
Sample 1									
1	85.8	53.1	29.9	24.5	20.7	16.7	12.6	10.0	9.7
2	91.7	54.6	30.2	26.0	22.0	17.0	12.8	10.0	9.5
3	92.3	55.3	31.2	26.6	21.6	17.5	13.1	10.6	9.7
5	96.8	58.7	32.7	27.8	23.2	18.0	13.9	10.9	9.3
9	86.7	46.5	27.0	24.0	21.2	17.2	13.3	10.5	9.5
Sample 2									
1	86.2	53.5	29.9	24.0	20.9	14.5	7.9	5.2	2.3
2	91.3	57.2	32.2	24.9	22.4	14.5	7.6	5.8	2.6
3	90.2	55.5	30.9	24.6	20.4	15.0	8.7	5.6	2.4
6	73.7	64.4	29.3	22.2	20.0	21.4	8.5	5.4	2.2
7	88.8	54.4	31.0	24.6	19.4	14.6	8.3	5.7	2.2

The conclusion from these experiments is that all CO saturated blood solutions are stable for up to 10 days if stored anaerobically at refrigerator temperatures, but only solutions containing 10% COHb or below are stable for longer periods.

4. Modifications to Analysis Method

All of the blood analyses carried out for the experiments described above were carried out using the current analysis method described in Annex A. However, it has been demonstrated above that this method is biased approximately 8% too low and has an average co-efficient of variation of 6.3%. The following modifications have been made to the method:

a. Use of a repeating pipettor to avoid loss of blood on pipette tip sidewalls.

b. Determination of vial volume from batch to batch.

c. Use of a 500 ppm CO in air standard instead of a 10 ul injection of pure CO. This improves reproducibility by eliminating the use of a small volume gas syringe.

d. Elimination of the headspace flush with helium prior to injection of the haemolyzing agent. This was originally introduced with a GC method using thermal conductivity detection. In this GC method, the CO elutes as a small peak on the tail of a much larger O_2/N_2 peak. By flushing with helium, much of the major air constituents were removed, allowing for a more sensitive and reproducible detection of the CO peak. However, using the current GC with a methanizer, flame ionization detection and backflush of the major air constituents (O_2 and N_2), the helium flush is a superflous step. Furthermore, there is a possibility of some CO being displaced from the blood and swept out by the helium.

e. Use of a reagent in the determination of total Hb which allows for the complete conversion of Hb species in the blood to CNMetHb within a reasonable time.

These modifications have been incorporated to form a new method which is outlined in Annex B. To test the reproducibility and accuracy of the new method, a series of 10 saturations each were carried out using the rotating tonometer and heated impinger methods to saturate blood. Degrees of saturation acheived were 100.9 $\pm$ 4.6% and 99.8 $\pm$ 4.8% respectively for each of these methods.

These results show that the new method is accurate and has a co-efficient of variation of less than 5%.

5. Comparison of Modified GC Method with Other Analysis Techniques

Eight human subjects were dosed with various levels of CO to validate the theoretical equations governing uptake and release of CO by the body. Blood samples were taken before, immediately after and 2.5 min after each dosing. Four subjects received two doses, three subjects received three doses and one subject received four doses. In addition, alveolar air samples were taken and analyzed for CO content. The blood samples were analyzed by both the new GC method and CO-oximetry. From these studies, 63 pairs of data comparing the COHb concentration determined by the GC and CO-oximetry methods and 50 pairs of data comparing COHb concentration measured by the GC method with estimates from alveolar CO concentrations were obtained, over the range of 0.5 to 20 vol% COHb.

The comparison of COHb concentration determined by GC with CO-oximetry is illustrated in Figure 11. The regression line is:

$$(COHb)_{CO-ox} = 0.925\ (COHb)_{GC} + 1.17$$

with an r^2 value of 0.979. However, comparison with the 1:1 line indicates that the CO-oximeter method overpredicts at low values of % COHb. To illustrate this point, Figure 12 shows a comparison plot for

FIGURE 11

Comparison of Gas Chromatography and CO-Oximetry for the Determination of % COHb on Blood

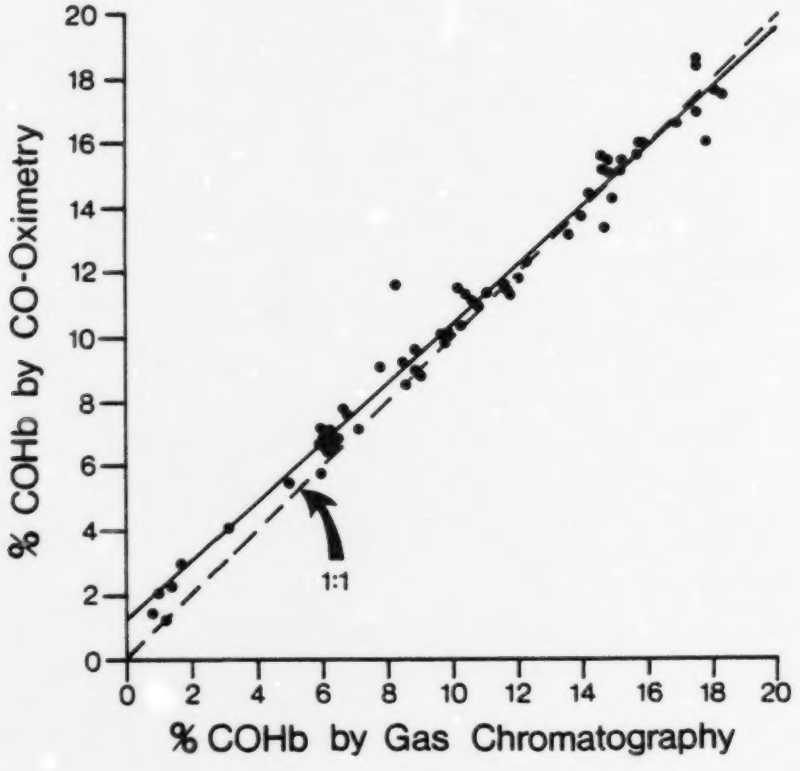
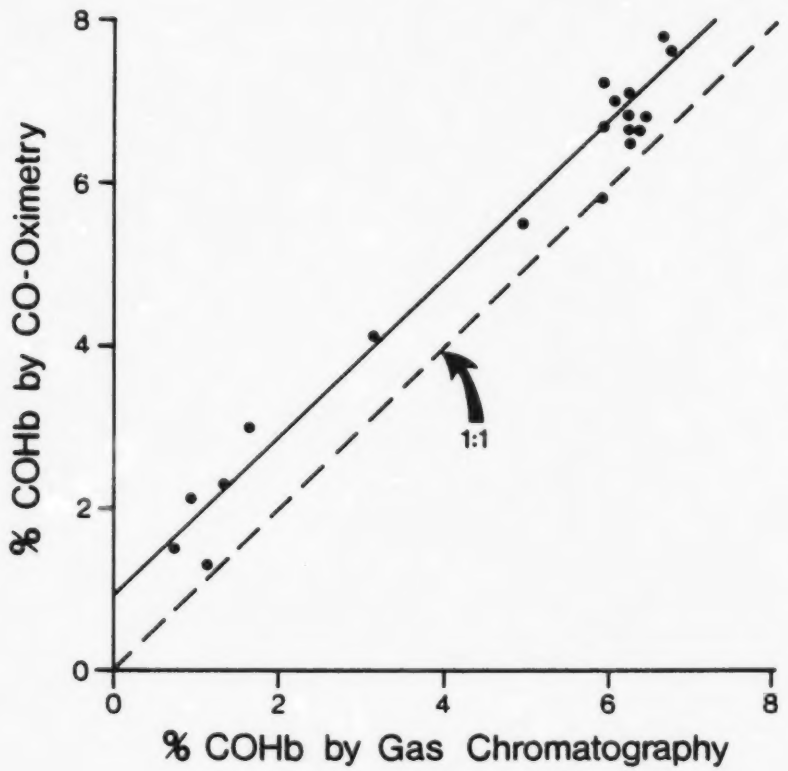


FIGURE 12

Comparison of Gas Chromatography and CO-Oximetry at Low COHb Concentrations



all values below 8% COHb. The bias of CO-oximetry at low COHb level is particularly evident. Similar bias has been reported in the literature (9). In contrast, the comparison of GC method with COHb estimated from alveolar CO concentrations is illustrated in Figure 13. The line has a regression of:

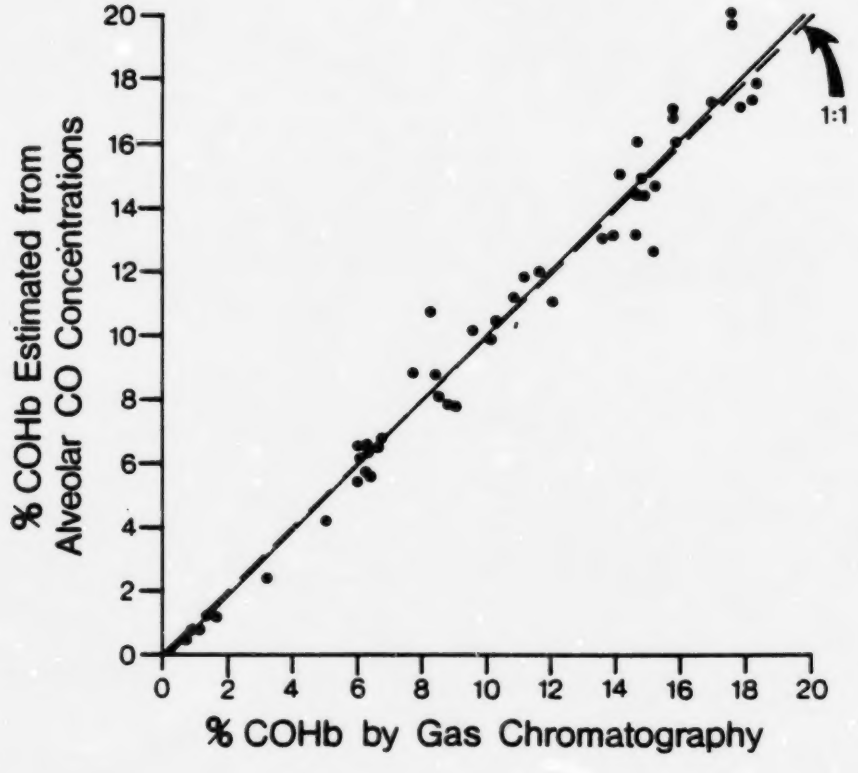
$$(COHb)_{Alv} = 1.025\ (COHb)_{GC} - 0.213$$

with an r^2 of 0.969 and is virtually indistinguishable from the 1:1 line.

These data illustrate the accuracy of the new GC method over the range of 0.5 to 20% COHb and, combined with the previously mentioned data for 100% COHb saturated blood, indicate that the method is accurate and precise over the whole range of COHb saturation. An additional observation from these data is that the method of estimating %COHb from alveolar CO measurements is the most accurate method for use in field studies.

FIGURE 13

Comparison of Gas Chromatography and Estimates from Alveolar CO Concentrations for the Determination of % COHb Concentrations in Blood.



CONCLUSIONS

The current method of determining the COHb concentration in blood by a GC technique has been critically evaluated. Table 11 summarizes the major sources of variation in the method and indicates where some variation can be reduced or eliminated. The major sources of bias in the method accuracy were found to be in the measurement of blood volume and in the separate determination of total Hb concentration in the blood, particularly at higher levels of CO saturation. The current method was estimated to have a co-efficient of variation (CV) of 6.2% over the range of 0.5 to 20% COHb concentration, and an average CV of 5.9% at a nominal 100% COHb concentration. The major source of variation in the method was found to be from small changes in GC flow rate and build-up of impurities on the GC column. Such variations can be minimized by a regular schedule of column bakeout and close control of carrier gas flow rate.

Comparison with spectrophotometric techniques, using both UV-visible spectrophotometry and a CO-oximeter showed that the GC method gave lower results by about 8%. This bias, as explained above, is due to two factors, the measurement of blood volume using single aliquot dispensing pipettors and to incomplete reaction using the current method of estimating total Hb. This also explains why CO saturation experiments gave average COHb concentrations of 93.7%.

It was found that a 500 ppm CO in nitrogen gas mixture flushed through the headspace of a vial gave a more reproducible standard for the analysis, with a coefficient of variation of 3.1% compared to a 6.7% CV for a parallel set of 10 ul CO standards.

Studies of long-term storage showed that COHb concentration, measured up to 100% saturation, remains constant for up to 9 days if the blood is stored anaerobically at refrigerator temperatures. Blood with 10% or less COHb saturation is stable up to 60 days.

Of the methods used to saturate blood and haemoglobin solutions with CO, an impinger or tonometer held at 37°C, through which moisture saturated CO gas is passed, gave the most effective results.

A modified procedure based on results obtained in this study is presented in Annex B. The method was validated with blood saturated with CO by both the rotating tonometer and impinger methods resulting in saturations of 100.9 $\pm$ 4.6% and 99.8 $\pm$ 4.8% respectively for the two methods. Thus, good accuracy and precision are acheived.

Comparisons of the modified procedure with CO-oximetry and estimates from alveolar CO measurements revealed that the new method is accurate over the range 0.5 to 20% COHb saturation, and also showed that CO-oximetry gives high results for low levels of COHb saturation. However, correlation with estimates from alveolar CO measurements was excellent and indicate the alveolar measurements would be the ideal technique for blood COHb surveys in the field.

TABLE 11

Sources of Method Variation and Error

Source	Effect
1. Vial Volume	Coefficient of variation 1.1%. Batch to batch variation is higher, thus volume determination on each batch required.
2. Volume of blood aliquot.	Large variation caused by blood sticking to sides of pipette. Can be avoided by using repeating pipettor.
3. Determination of %Hb.	In standard method using Drabkin's solution, conversion of COHb to CNMetHb is slow at high concentrations of COHb. Thus, determination after recommended time of 10 min. can result in low %Hb values. Problem solved by using higher $K_3Fe(CN)_6$ concentration in reagent.
4. Acidity of CO releasing agent.	CO fully liberated with normal high acidity reagent.
5. Time of reaction after addition of releasing agent.	No effect observed at times from 2 min to 1 hr.
6. Ambient temperature and pressure.	Results corrected to STP.
7. GC temperature and flow rate changes.	Flow rate changes can cause considerable variation in observed peak area. Require daily checks of flow rate.
8. Catalyst efficiency.	Operating temperature of 400°C gives complete conversion of CO to CH_4.
9. CO losses during transfers and manipulations of the analysis method.	No significant losses observed. Overall contribution to method coefficient of variation is estimated to be less than 2%.
10. Injections to GC.	Using gas mixtures containing known concentrations of CO, it was found that the GC analysis was accurate over the range of 10 to 500 ppm CO in a headspace sample (equivalent to the amount of CO liberated from 0.5 ml of blood containing from 0.25 to 10 vol. % of COHb).

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ANNEX A

THE DETEMINATION OF CARBON MONOXIDE IN TISSUE AND BLOOD BY GAS CHROMATOGRAPHY

Introduction

The method described here for the quantitative determination of carbon monoxide in tissue and blood depends on the release of carbon monoxide from the haemoglobin by a chemical releasing agent, and the measurement of the resulting vapour by gas chromatography.

Any carbon monoxide present in a 0.5 ml aliquot of tissue/borate supernatant, or blood, contained in a sealed 20 ml reaction vial, is reacted with 0.5 ml of releasing agent for 4 minutes at room temperature. A 1.0 ml headspace vapour sample is removed and analyzed by gas chromatography in comparison with a carbon monoxide standard.

Procedure

1. Prepare all standards/samples in duplicate.
2. STANDARD: Pipette 0.5 ml borate buffer in a 20 ml reaction vial.
3. Stopper the flask(s).
4. Insert a 20-gauge needle through the stopper, and attach another 20-gauge needle to the helium flush apparatus.
5. Flush the flask and liquid thoroughly with gaseous helium to remove all the air (the more thoroughly this is carried out, the sharper the resulting peak heights will be).
6. Transfer pure carbon monoxide from the supply cylinder to the small plastic bag and seal the opening with tape to ensure purity of the sample.
7. Using a 100 ul gas tight syringe, draw 10 microlitres from the bag and inject it through the septum of the reaction vial. Seal the puncture in the bag with tape.
8. Prepare the releasing agent by mixing potassium ferricyanide and haemolyzing agent in a 1:1 ratio.
9. Inject 0.5 ml of this solution through the stopper, using a disposable syringe.
10. Agitate the vial vigorously.
11. Prepare the 2.0 ml injection syringe by fastening a 20-gauge needle and inserting the plunger so as to remove all air from the syringe.

12. Draw approximately 1.5 ml of headspace vapour into the syringe, inject it back into the vial and repeat this three times to obtain a homogenous sample. Finally withdraw 1.0 ml of the headspace vapour and adjust the volume adapter. Remove the syringe and needle from the septum and then remove the needle from the syringe.

13. Immediately insert the syringe tip into the GC injection port and inject the contents of the syringe in the GC sample loop in a smooth and continous sequence.

14. Area of the standard peak should read no less than 9600 units.

Operation of the CARLE 211M

1. Helium carrier gas flows at all times, 60 psi.

2. Synthetic air - 20 psi and Hydrogen at 30 psi.

3. Light flame, turn methanizer switch to on (leave dial set at 40 at all times) and put event sequence at start position.

4. Allow methanizer and FID to stabilize for approximately one hour.

5. Parameters for the GC/integrator follows:

Column Temp	75°C	Report - Area %	Slope Sens - 0.1 (.03)
Methanizer	400°C	Delay - 0.5	Attenuation - 1
Range	10	Timer - 5 min	
Output	1 (4)	Area reject - off	
		Chart speed - 1 cm/min	
		Chart - Auto	

Numbers in brackets represent parameters when low COHb containing samples are being analyzed.

Calculation and Reporting of CO Saturation

If CO detection is nil:

1. Report as nil.

2. It is not necessary to perform a haemoglobin detemination.

If CO detection is positive:

1. Determine the haemoglobin concentration of each sample using the cyanmethaemoglobin method.

2. Report the %CO saturation as:

a. if less then 2.0%, report as 2.0% CO saturati

b. if greater than 2.0%, report to one decimal pl e.

Calculation

Note the temperature and pressure during the analysis period.

1. Average the peak heights of the CO standards.

2. Average the peak heights of both injections of ach tissue/blood sample.

3. Convert the temperature to degrees absolute:

$$°C + 273 = °A$$

4. Using a conversion chart report the pressure in Torr as opposed to millibars.

5. Apply the figures obtained in steps 1 through 4 in the following formula to obtain %CO saturation.

$$\frac{D}{S} \times \frac{P}{T} \times \frac{53.2}{Hb} = \% \text{ CO saturation}$$

Where:

D = peak height of sample
S = peak height of standard
P = barometric pressure in Torr
T = room temperature in degrees absolute
Hb = haemoglobin in gram %
53.2 = constant used for standard of 10 ul of CO and 0.5 ml aliquot at a GC attenuation for the sample of 10 x 1.

REAGENTS

1. 20% W/V Potassium Ferricyanide

a. Prepare every 3 months and store in refrigerator.

b. Weight 100 grams of $K_3Fe(CN)_6$.

c. Dissolve in a beaker with approximately 250 ml distilled water - use a magnetic stirring bar.

d. Pour into a 500 ml volumetric flask.

e. Rinse the beaker and top the volumetric flask to the mark with distilled water.

f. Transfer the solution to a dated storage bottle and place in a refrigerator.

2. Haemolyzing Agent

a. Using a volumetric pipette, measure 10 ml of Triton X 100 into a 500 ml volumetric flask.

b. Add about 300 ml distilled water.

c. Mix slowly with a magnetic stirrer to avoid foaming until Triton X is in solution and then removed the stirring bar.

d. Add slowly 85 ml conc HCl (measure with a graduated cylinder).

e. Top up to the mark with distilled water

f. stable indefinitely when stored in a "Nalgene" bottle at room temperature.

3. Borate Solution (pH 8.2)

a. Weigh out 6.18 grams of Boric Acid and place in a 500 ml volumetric flask.

b. Add 55 ml of 0.2 N Sodium Hydroxide into the 500 ml volumetric flask and stir with magnet until dissolved.

c. Top up to the mark with distilled water. (Remove magnet before topping up.)

d. Stable indefinitely when stored at room temperature.

4. 0.2N Sodium Hydroxide

a. Weigh out 8.0 grams of 100% NaOH. Speed is essential because of the tendancy of NaOH to absorb water vapour.

b. Dissolve the NaOH in 1000 ml of distilled water in a volumetric flask.

c. Stable indefinitely when stored at room temperature.

ANNEX B

THE DETERMINATION OF THE AMOUNT OF CARBON MONOXIDE BOUND TO HAEMOGLOBIN IN BLOOD BY GAS CHROMATOGRAPHY

1. Introduction

In this method, carbon monoxide is released from carboxyhaemoglobin in blood by a chemical releasing agent and subsequently analysed in the resulting vapour by gas chromatography (GC). A known amount of blood, normally 0.5 cm^3, is measured into a vial. The vial is sealed, 0.5 cm^3 of releasing agent added, and any carbon monoxide present allowed to evolve into the vial head-space over a 5-minute period at room temperature. A 1.0 cm^3 sample of the head-space vapours is taken and analysed by GC in comparison to a carbon monoxide standard.

2. Apparatus

a. Reaction Vials, nominal sealed capacity 23 ml, with rubber septa and aluminum crimp seals.

b. Eppendorf repeating pipette.

c. Disposable syringes, 5 cm^3 capacity.

d. Gas syringe, 2.0 cm^3 capacity.

e. Carle 211 GC fitted with flame-ionization detector and methanizer unit, 10 port solenoid valve and 4 port solenoid valve. Columns: 7 ft by 1/8 in silica gel, 60/80 mesh; 2 x 3 ft by 1/8 in molecular sieve 5A, 80/100 mesh.

f. Hewlett-Packard 3380A recording integrator.

g. Scientific Products Model S8220 Vortex Mixer.

h. Fisher Model 74D Hemophotometer.

i. 10 ml cuvettes for hemophotometer.

j. Sahli pipette - 20 ul capacity.

k. Rotating thermostated tonometer (for blood saturation).

3. Reagents and Chemicals

a. Gas mixture, 500 ppm CO in nitrogen, primary standard grade.

b. Gas mixture, 1% CO in helium.

c. Carbon monoxide, C.P., 99.5% min.

d. Helium, high purity.

e. 20% w/v Potassium Ferricyanide Solution

i) Weigh 100 g of $K_3Fe(CN)_6$ to nearest 1 mg.

ii) Dissolve in approximately 250 cm^3 of deionized water in a beaker (magnetic stirring is required).

iii) Pour into 500 cm^3 volumetric flask.

iv) Rinse the beaker and make up to mark in flask with deionized water.

v) Transfer solution to a storage bottle, label and date, and place in refrigerator.

vi) Prepare every 3 months.

f. Haemolyzing Agent

i) Measure 10 cm^3 of Triton X-100 into a 500 cm^3 volumetric flask using a volumetric pipette.

ii) Add about 300 cm^3 of deionized water.

iii) Mix slowly with a magnetic stirrer to avoid foaming until the Triton X-100 is in solution; remove stirring bar.

iv) Slowly add 85 cm^3 concentrated hydrochloric acid (measured with graduated cylinder).

v) Make up to mark with deionized water.

vi) Store in bottle at room temperature (stable indefinitely).

g. pH 8.2 Borate Buffer Solution

i) Weight out 6.18 g of boric acid and place in 500 cm^3 volumetric flask.

ii) Add 55 cm^3 of a 0.2 N sodium hydroxide solution into the flask and stir with magnet until all ingredients are dissolved.

iii) Make up to mark with distilled water (remove magnet prior to making up).

iv) Store in bottle at room temperature (stable indefinitely).

h. 0.2 N Sodium Hydroxide Solution

i) Weight out 8.0 g of sodium hydroxide (1).

ii) Dissolve sodium hydroxide in 1000 cm^3 of deionized water in a volumetric flask.

iii) Store in a bottle at room temperature (stable indefinitely).

i. Cyanating Agent

i) Weigh out 1.000 g of potassium ferricyanide, 0.050 g of potassium cyanide and 0.0140 g of potassium dihydrogen phosphate and place in a 1000 cm^3 volumetric flask.

ii) Add 200 cm^3 of deionized water and stir until all crystals are dissolved. Make up to 1000 cm^3 with deionized water.

iii) Store in a dispenser bottle, set to deliver 5 ml aliquots, at room temperature (stable indefinitely).

4. Procedure

All standards and samples must be prepared in duplicate.

a. Standards (gas)

i) Pipette 0.5 ml pH 8.2 borate buffer solution into a reaction vial.

ii) Seal vial.

iii) Insert 20 gauge needle through the septum and attach another 20 gauge needle to the 500 ppm CO in N_2 mixture.

iv) Flush the vial thoroughly (at least 10 volume exchanges (250 cm^3)) with the gas mixture.

v) Prepare releasing agent by mixing 20% potassium ferricyanide solution and haemolyzing agent in a 1:1 ratio.

vi) Inject 0.5 cm^3 of this solution into the vial using a 5 cm^3 disposable syringe.

vii) Agitate the vial thoroughly using the vortex mixer.

viii) Attach 20 gauge needle to 2.0 cm^3 gas syringe. Insert needle into vial headspace and draw approximately 1.0 cm^3 of headspace vapour into the syringe. Inject back into the vial and repeat several times to obtain a homogeneous sample. Finally, withdraw 1.0 cm^3 sample.

ix) Remove syringe and needle from septum and then remove needle from syringe (2).

x) Immediately insert syringe tip into GC inlet line and flush sample loop (4).

xi) Press start button to initiate sample flush onto GC columns. Start integrator after second valve switch.

xii) Area of the peak is proportional to the headspace CO content:

$$\text{Headspace content} = \frac{500}{10^6} \times v \text{ ml}$$

Where: v = vial volume (ml) - 1.0 ml.

b. Samples

i) Using the Eppendorff repeater pipette, draw approximately 2.5 ml of well-mixed blood into the sample reservoir. Discard the first volume of blood.

ii) Pipette appropriate volume (for routine analyses use 0.5 cm^3) of well-mixed sample into a reaction vial and seal vial.

iii) Add 0.5 cm^3 of releasing agent (prepared as in step a.(v)) to vial, as described in a.(vi) above.

iv) Mix contents using vortex mixer.

v) Allow to stand for at least 5 minutes.

vi) Sample headspace and inject to GC as outlined in steps a. (viii) to a. (xi) above.

c) Carle 211 Operation

i) Helium carrier gas flow-set at 30 cm^3/min for analysis cycle, 40 cm^3/min for back flush cycle, as outlined in valve sequencing literature. All flowrates corrected to column temperature and for water vapour pressure in bubble flow meter.

ii) FID flows, air and hydrogen (through methanizer) according to manual.

iii) Light flame, and put event sequencer to start position. Methanizer should be left on at all times with carrier flow on.

iv) Allow FID to stabilize for 1 hour.

v) Parameter for GC and integrator are:

Column temp,	75°C	Report, area %
Methanizer,	400°C	Delay ,0.5 min
Range,	1 or 10	Timer, 5 min
Output,	1 or 4 (5)	Area Reject, off
		Chart Speed, 1 cm/min auto
		Slope Sensitivity, 0.1 or 0.03
		Attenuation, 1

d. Determination of Haemoglobin Content

i) Perform all samples in duplicate.

ii) Measure 5.0 cm^3 of cyanating reagent into 10 cm^3 cuvette.

iii) Measure 20 ul of well-mixed sample using a Sahli pipette and inject into the cyanating reagent. Mix well using the vortex mixer.

iv) Let stand for at least 10 minutes (6).

v) Zero the hemophotometer using cyanating reagent and calibrate using the cyanmethaemoglobin standard (7).

vi) Measure the sample. The hemophotometer gives readout directly in gram percent Hb.

e. Saturated Blood Checks

i) Transfer 5 cm^3 of blood to a 250 cm^3 tonometer flask using a graduated cylinder.

ii) Place in thermostated rotating tonometer and saturate with pure CO gas for 30 min at a flowrate of 200 cm^3/min.

iii) Flush flask in tonometer with 1% CO in helium mixture for 5 min.

iv) Determine degree of CO saturation in duplicate using the procedure outlined in Section (b) above on a 100 ul aliquot of saturated blood.

v) This procedure should be carried out every three months routinely. Special projects may require more frequent checks.

f. Calculation of Results

i) Note ambient temperature and pressure during the analysis period in Kelvins and mmHg.

ii) Average the peak areas of the standard and sample determinations.

iii) Calculate %COHb concentration in blood using the equation

$$\%COHb = \frac{A}{S} \times \frac{P}{T} \times \frac{500}{10^6} \times \frac{H}{1.35} \times \frac{100}{(Hgb)} \times \frac{273}{760} \times \frac{100}{V}$$

Where: A = peak area of unknown sample
S = peak area of standard
P = ambient pressure in mmHg
T = ambient temperature in Kelvins
H = headspace volume in vial. Calculate by taking vial volume of current batch and subtracting 0.5 ml for the releasing agent and then subtracting the volume of blood measured (normally 0.5 ml).
Hgb = blood haemoglobin concentration in g/100 cm^3
V = volume of sample taken

iv) If CO detection is nil, report as nil, if CO is detected, report as %CO saturation to one decimal place.

NOTES

1. Speed is essential because of the tendency of sodium hydroxide to absorb atmospheric water vapour.

2. Determine vial volume in duplicate for each batch of 100 vials used as vial volume can vary by as much as 8% between batches.

3. All operations between removing sample from the vial headspace to the GC inlet must be carried out as quickly as possible to minimize possible gas exchange with the ambient atmosphere.

4. The flushing procedure must be carried out slowly and evenly to avoid back diffusion of ambient air, and injection to the GC should be started immediately after flushing is completed.

5. Range and output of GC are set depending on peak height (and hence COHb concentration). If high COHb concentrations are expected, other settings may be used, however, corrections may be required as attenuations may not be exact multiples. If an unknown yields an offscale peak, the sample should be repeated, either using a lower blood volume, or at a higher GC attenuation.

6. This reaction time is suitable for most of the analyses that will be encountered. However, high concentrations of COHb have been found to retard the formation of cyanmethemoglobin. Samples containing higher than 25% COHb should be allowed to stand for at least 60 min.

7. The absorbance of the cyanating agent and the diluent used in the standard are essentially zero at the wavelength used, despite differences in potassium ferricyanide concentration of each solution.